INVESTIGATING THE POTENTIAL USE OF VIRUS TECHNOLOGY TO FURTHER OUR UNDERSTANDING OF FLORAL INDUCTION AND ITS APPLICATION IN PLANT BREEDING PROGRAMMES.

by

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Declaration

This thesis is the result of my own work which was performed during the period of my PhD registration. None of this work has been presented for another degree.

Femi David Akande

Summary

Flowering Locus T (*FT*) plays a pivotal role in floral induction. It integrates the inputs from a complex network of flowering signalling pathways. Flowering is an efficiently orchestrated event that occurs in a plant at a particular time to ensure maximum reproductive success. It has been suggested that the FT protein is a long- distance mobile floral stimulus. In this report studies with a mutant version of *FT* (m*FT)* which had the start codon replaced with a stop codon to generate a non-translatable *FT* indicated that the mRNA was also capable of long distance movement although its physiological function as a floral stimulus was inhibited.

Gene function study of *FT* and *FT* orthologues on brassica, tobacco, tomato and potato using the plant virus expression vector Potato Virus X (PVX) generated some interesting findings. In Short day Maryland Mammoth tobacco plants the overexpression of the *Arabidopsis FT* under non-inductive Long day condition induced early flowering while the *mFT* and mock control remained in the vegetative stage. In short day potato, it did not seem to have an effect on tuberization as only one from five of the inoculated plants tuberized. In brassica (broccoli) the effect on flowering time was inhibited due to Virus-induced Gene Silencing (VIGS) but the tomato *FT* (*SP6A*) had an effect on flowering time.

In tomato, the overexpression of the *Arabidopsis FT* and *FT*- orthologues from tomato induced early flowering but the difference in flowering time in comparison to the controls was only a few days. Phenotypical and morphological changes such as seed production and lateral side shoot development were caused by expression of the target genes. The exact mechanism of action of these genes in the control of seed production and lateral side shoot development is unclear.

Abbreviations

Aa	Amino acid
AP1	*APETALA 1*
bp	Base pair
CCA1	*CIRCADIAN CLOCK ASSOCIATED 1*
CCs	Companion cells
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
CO	*CONSTANS*
Col	Columbia
CP	Coat protein
DTT	Dithiothreitol
CRY	Cryptochromes
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphospahte
dpi	Days post –inoculation
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
EF1α	*Elongation factor 1 alpha*
FD	*Flowering Locus D*
FLC	*Flowering Locus C*
FT	*Flowering Locus T*
FUL	Fruitful
GA	Gibberellin
g	Grams

g	Relative centrifuge force
GFP	Green Fluorescent Protein
GI	*Gigantea*
GUS	β-glucuronidase
HD1	*Heading date 1*
Hd3a	*Heading date 3a*
LB	Luria-Bertani
LD	Long days
LFY	LEAFY
M	Molar
MFT	MOTHER OF FT and TFL1
MM	Maryland Mammoth
$MgCl_2$	Magnesium Chloride
Mins	Minutes
MiR156	MicroRNA 156
MiR159	MicroRNA 159
MiR172	MicroRNA 172
MiRNAs	MicroRNA
mM	Milimolar
NaCl	Sodium chloride
ORF	Open reading Frame
PCR	Polymerase chain reaction
PEBP	Phosphatidylethanolamine binding protein
PHY	Phytochromes
PVX	Potato virus X
RdRP	RNA- dependent RNA Polymerase
R	Red light
RIF	rifampicin

RMA	RNA Mobility Assay
RNA	ribonucleic acid
rpm	revolutions per minute
s	seconds
SAM	Shoot Apical Meristem
SD	Short Days
SEs	Sieve Elements
SFT	*SINGLE-FLOWER TRUSS*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SP	*SELF PRUNING*
SPEC	spectinomycin
TSF	*TWIN SISTER OF FT*
VIGS	Virus-induced Gene Silencing
Vir	Virulence
WT	Wild Type

Chapter 1

General Introduction

Chapter 1: General Introduction

1.1 The regulation of flowering

Flowering is the transition of a plant from the vegetative to the reproductive phase and is one of the major phase changes during a plant's life cycle. It has been established that multiple interconnected signalling pathways are involved in the regulation of flowering times in response to environmental and endogenous factors (Bernier *et al*., 2005). Flowering is influenced by seven distinct pathways. These are the photoperiodic, autonomous, vernalization, GA, light quality, ambient temperature and microRNA pathways (Wigge 2011) ; Aukerman *et al*., 2003). The majority of these pathways are integrated by the floral integrator gene *FLOWERING LOCUS T* (*FT)* which is a key inducer of flowering. The FT protein has been shown to be a component of the mobile florigen and it is important in inducing flowering in many plant species (Wigge 2011). Movement of the FT protein has been well researched and documented in recent years. The accepted theory for the induction of flowering is that after induction of *FT* gene expression by CONSTANS (CO) protein in the leaf, the FT protein moves from the leaf to the shoot apical meristem (SAM) via the phloem and induces flowering (Corbesier *et al*., 2007).

Arabidopsis thaliana, a facultative long day plant has been used as a model for molecular and genetic study of flowering pathways. Numerous flowering time mutants have been generated either through induced mutagenesis or natural variation. For example mutations within the target genetic loci of the genes involved in the control of flowering: *gi (gigantea), co (constans), cry2 (cryptochrome), flowering locus d (fd)* and *flowering locus (ft)* were reported to flower later than wild-type plants under LDs. This indicated that the loci was involved in the positive regulation of flowering whilst mutations causing earlier flowering suggested the disrupted genes may be required for the repression of flowering (Komeda 2004). The GI-CO-FT proteins act in concordance to regulate floral induction. *CO* mRNA expression differs between LDs and SDs. In LDs, the level of GI peaks and in turn this causes the expression of *CO* mRNA (Fig 1.1a). Quantitative levels of CO protein gradually increases and peaks between 10hrs and 12hrs after dawn. The stability of this protein is dependent on mRNA expression and light. CO protein is stabilized by the action of PHYTOCHROME A (PhyA), CRYPTOCHROME (CRY1) and CRY2 (Fig 1.1b). CO protein is rapidly degraded in the dark hence plants growing under SDs will not accumulate the protein. In the morning, PhyB negatively regulates CO while at night CO degradation depends on the presence of

SUPRESSOR OF PHYA-105- 1 (SPA1), SPA3 and SPA4 (Fig1.1b). *FT* mRNA transcription is induced by the direct accumulation of CO protein and regulation of *FT* by *CO* has been suggested to occur through an interaction of CO and the CCAAT-box binding protein factor with the 5' UTR region of *FT* (Ben-Naim *et al*., 2006).

FT protein moves through the phloem's sieve plates and sieve elements and on getting to the cells within the SAM, FT protein interacts with the FD bZIP transcription factor forming a complex which results in direct upregulation of *SOC1* mRNA (Corbesier *et al*., 2007); (Jackson 2009; Wigge *et al.*, 2005; Wigge 2011). The SOC1 protein forms a complex with AGAMOUS-LIKE 24 (AGL24) which translocates to the nucleus where it binds the *LFY* promoter to induce *LFY* expression (Lee *et al*., 2008). The FT/FD complex could also induce the expression of floral meristem identity genes such as *AP1* (Boss *et al*., 2004; Kaufmann *et al*., 2010) and *FUL* (Corbesier *et al.*, 2007; Turck *et al*., 2008).

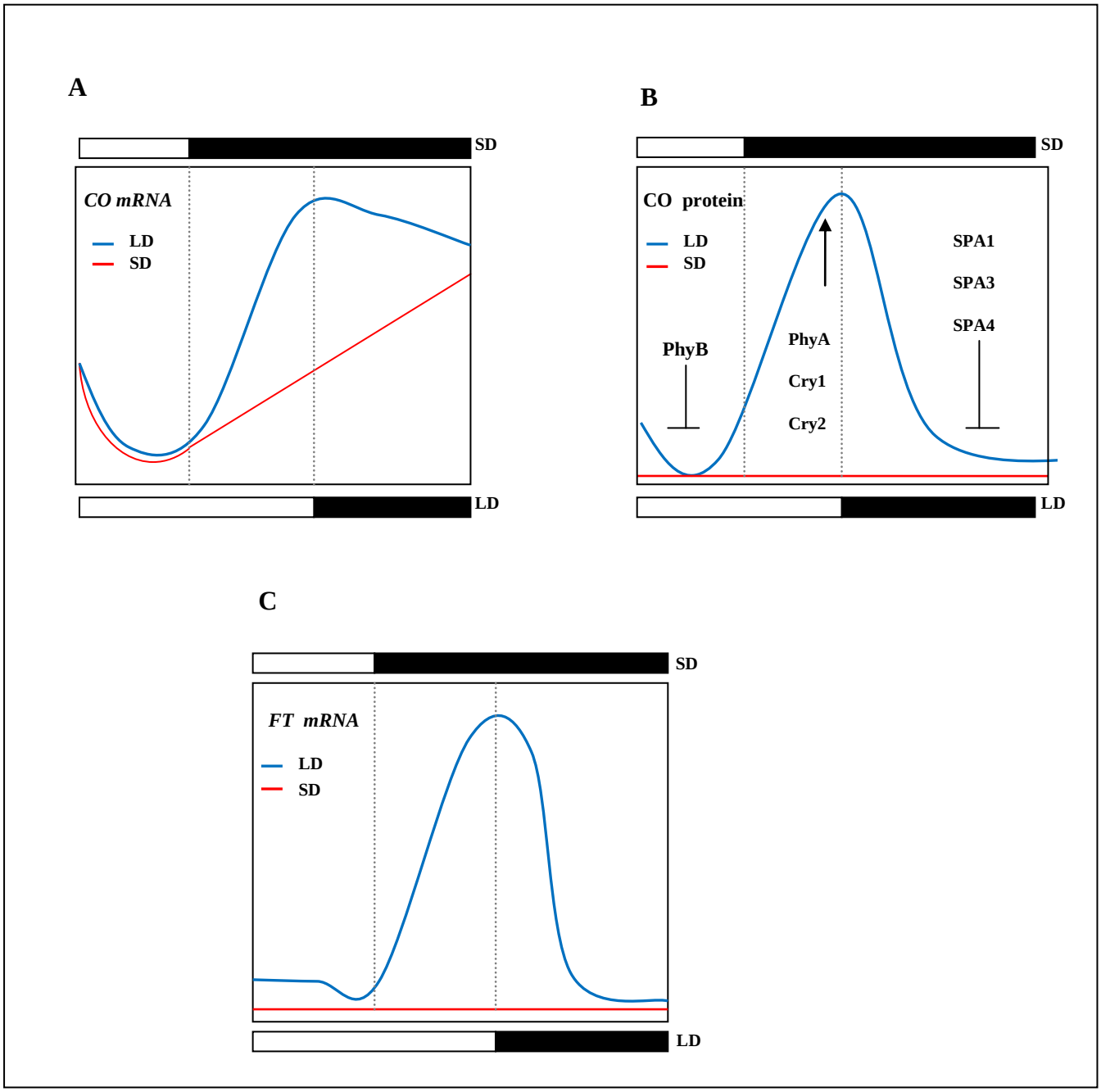


Fig 1.1 Circadian expression of key components in floral regulation. (a) *CO mRNA* expression exhibits a biphasic curve pattern under LDs. (b) Accumulated CO protein is dependent on both mRNA expression and light. Quantitative level of CO protein is stabilized by CRY1, CRY2 and PhyA towards the end of LDs. It is negatively regulated by PhyB in the early morning and at night CO degradation is mediated by the presence of SPA1, SPA3 and SPA4. This also counteracts early peak in CO which could have been caused by high CO mRNA levels towards the end of the night. (c) *FT* mRNA expression under LDs is triggered by the direct action of accumulated CO protein. *FT* mRNA / FT protein move from the leaf to the shoot apical meristem (SAM) through the phloem's sieve plates and sieve elements. At the SAM the FT protein interacts with the FD bZIP transcription factor forming a complex which results in the direct upregulation of *SUPPRESSOR OF CONSTANS 1* (*SOC1*) mRNA. SOC1 in turn forms a complex with AGAMOUS-LIKE 24 (AGL24) which translocates to the nucleus where it binds the *LEAFY* (LFY) promoter to induce *LFY* expression which ultimately induces the development of floral primordia**.** The FT/FD complex could also induce the expression of floral meristem identity genes such as *APETALA1*(*AP1*). Arrows indicate activation and T-bars represents inhibition.

1.1.1 Pathways involved in the promotion and regulation of flowering

The transition to the reproductive phase in plants is regulated by an interconnected network of signalling pathways. In the model plant *Arabidopsis thaliana* many molecular and genetic approaches have been applied to study pathways involved in the regulation of flowering (Boss *et al*., 2004). The pathways involved in flowering regulation are shown in Fig 1.2 below. The key genes involved in these pathways include *FT*, *FLOWERING LOCUS C (FLC), CONSTANTS (CO), GIGANTEA (GI), LEAFY (LFY), F-BOX PROTEIN1 (FKF1), TERMINAL FLOWER1 (TFL1), FRUITFUL(FUL), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1(CCA1), GIBBERELLIN INSENISTIVE DWARF 1(GIDI), TWIN SISTER OF FT (TSF)* and *APETALA1* (*AP1*) (Wigge 2011; Hirano *et al*., 2008; Turck *et al*., 2008).

FLC plays a pivotal role in the regulation of flowering (Yant *et al*., 2009). The repression of *FLC* by the vernalization and autonomous pathways promote flowering by relieving the inhibition of *FT* and *SOC1* by *FLC* (Lee *et al*., 2010; Moon *et al*., 2005). The flowering network pathway acts as a complex control system in the sense that the repressor and integrator genes coordinate the flowering response to changes in both endogenous and environmental cue (Boss *et al*., 2004).

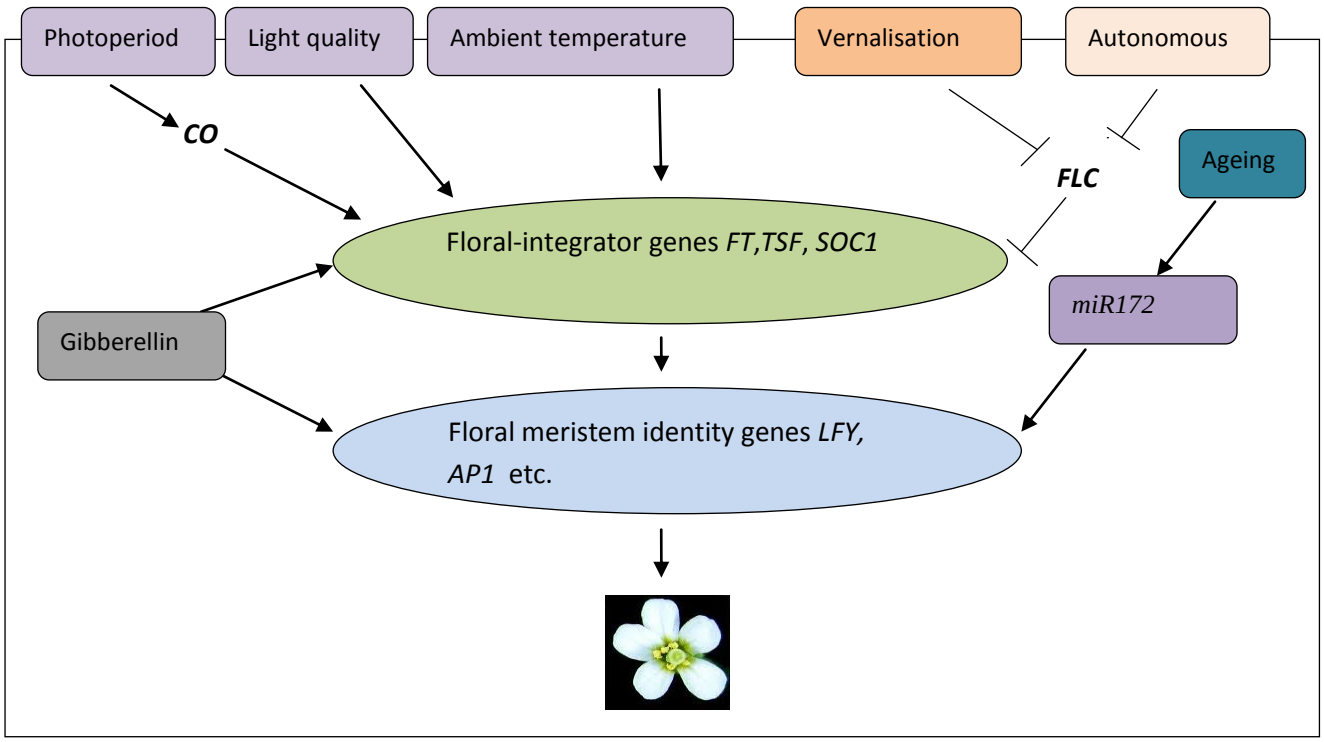


Fig 1.2 The main pathways involved in the control of flowering in *Arabidopsis thaliana.* Arrows indicate activation and T-bars represents inhibition. *CO* represents *CONSTANS*, *GI* represents *Gigantea*, *FT* represents *FLOWERING LOCUS T*, *TSF* represents *TWIN SISTER OF FT*, *SOC1* represents *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*, *LFY* represents *LEAFY*, *AP1* represents *APETALA1*, *miR172* represents microRNA172 and *FLC* represents *FLOWERING LOCUS C.*

1.1.1.1 The Photoperiodic Pathway

The photoperiodic control of floral induction is important because the perception of changes in day length is the major way for plants to sense the ever changing season. *Arabidopsis thaliana* shows a strong photoperiodic response in the onset of flowering. Flowering occurs much earlier under LD of 16h light than under SD of 10hr (Searle *et al*., 2004). In the model plant *Arabidopsis thaliana*, numerous flowering regulators involved in the photoperiodic pathways have been identified. They include ZEITLUPE (ZTL) which is a blue light receptor, the red and far-red light absorbing phytochromes (PHYA-E), UV/ blue light absorbing cryptochromes (CRY1,2) and components of the circadian clock which include *LHY, CCA1, TOC1, GI.* Key regulatory factors include *FT, CO, TSF, FKF1, CDF1, FWA* and *DAY NEUTRAL FLOWERING* (*DNF*) (Morris *et al.*, 2010); (Thornber *et al*., 2006); (Jackson 2009; Mockler *et al*., 2003; Mas *et al.*, 2009). The photoperiodic pathway is initiated in the leaves with the perception of light by the red/far-red light-receptors phytochromes *(PHYA-E)* and the blue/UV-A light receptors cryptochromes (CRY1 and 2) (Clack *et al.*, 1994; Lin *et al*., 2005; Quail 2002). These photoreceptors are involved in mediating light input to the circadian clock (Guo *et al*., 1998). Photoreceptors interact to entrain the circadian clock to a 24h period. In plants, the circadian clock regulates a diverse range of biological processes and it is the plant's endogenous time keeper (Halliday *et al*., 2003). Several genes have been identified as components of the circadian clock and they show high levels of similarity and functional redundancy (Nakamichi 2011). PHYB binds to PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) and causes the up-regulation of both *CCA1* and *LHY* expression which peak early in the morning shortly after dawn. ZTL interacts with TOC1 by targeting the protein for degradation via the 26S proteosome (Mas *et al*., 2003).

The clock regulates *CO* transcription positively through the GI/FKF1 complex which promotes the degradation of the repressor CDF1. CDF1 down regulates the expression of *CO* by binding to its promoter region (Imaizumi *et al.*, 2003). The transcription level of *GI* and *FKF1* peaks much later in the day which leads to the degradation of CDF1 at that time of the day thus allowing the induction of *CO* transcription towards the end of a LD (Sawa *et al*., 2007; Salazar *et al*., 2009). The clock's regulation of *CO* results in a circadian rhythm with the *CO* mRNA rising in abundance between 10-12 hrs after dawn.

CO is stabilized by blue and far red light through *PHYA* and *CRY1/2* and the accumulation of CO protein induces the transcription of floral integrator gene *FT* (Samach *et al*., 2000; Turck *et al*., 2008; Cerdan *et al*., 2003; Suarez-Lopez *et al*., 2001; Valverde *et al*., 2004). Regulation of *FT* by *CO* has been suggested to occur through an interaction of CO and the CCAAT-box binding protein factor with the 5' UTR region of *FT* (Ben-Naim *et al*., 2006).

CO is expressed primarily in the phloem companion cells of the leaf where it induces the expression of *FT* (An *et al*., 2004). FT protein moves through the phloem's sieve plates and sieve elements and on getting to the cells within the SAM, FT protein interacts with the FD bZIP transcription factor which results in direct upregulation of *SOC1* mRNA (Corbesier *et al*., 2007; Jackson 2009; Wigge *et al*., 2005; Wigge 2011). The SOC1 protein forms a complex with AGAMOUS-LIKE 24 (AGL24) which translocates to the nucleus where it binds the *LFY* promoter to induce *LFY* expression (Lee *et al*., 2008). The FT/FD complex could also induce the expression of floral meristem identity genes such as *AP1* (Boss *et al*., 2004; Kaufmann *et al*., 2010 and *FUL* (Corbesier *et al*., 2007; Turck *et al*., 2008). Both pathways lead to floral induction in the developing primordial (Fig 1.3).

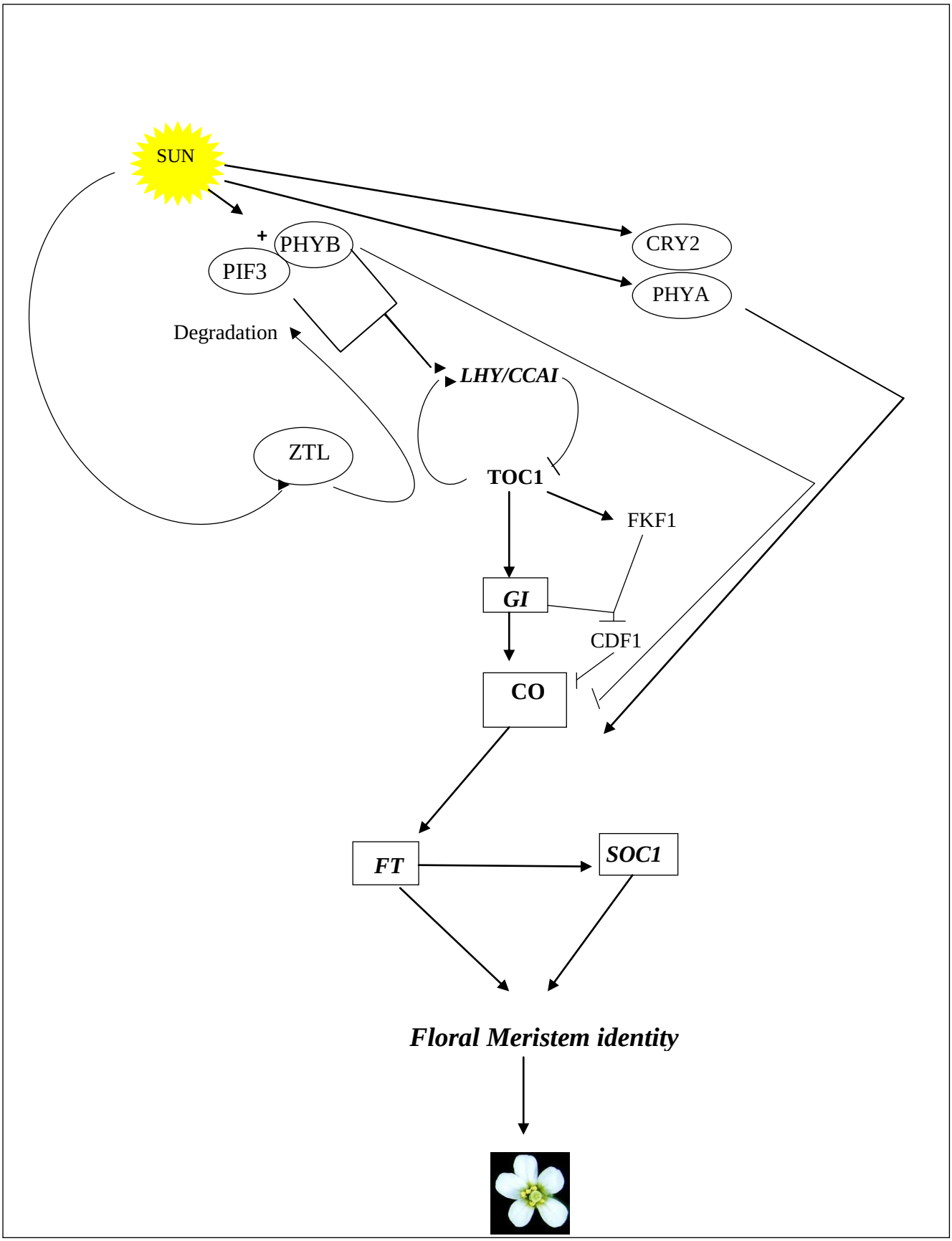


Fig 1.3 A schematic diagram of the photoperiodic pathway in *Arabidopsis thaliana.* Arrows indicate activation and T-bars represents inhibition. CRY2 represents cryptochrome 2, PIF3 represents PHYTOCHROME-INTERACTING FACTOR 3, PHY represents Phytochromes, *CCAI* represents *CIRCADIAN CLOCK ASSOCIATED 1, LHY* represents *LEAFY*, *TOC1* represents *TIMING OF CAB EXPRESSION 1*, FKF1 represents F-Box protein 1,CDF1 represents Cycling DOF factor 1, ZTL represents ZEITLUPE, *SOC1* represents *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*, *FT* represents *FLOWERING LOCUS T* and *GI* represents *Gigantea*.

1.1.1.2 microRNA pathways affecting flowering

It has recently been discovered that miRNAs play an important role in developmental transition. MicroRNAs are non- coding endogenous small RNAs that have a role in the regulation of flowering (Bartel 2004). The transition from juvenile to adult phase is mediated by *miR156* with decreasing quantitative levels over time (Fig 1.4). *miR156* is also involved in the down-regulation of SPL expression in the phloem companion cells which ultimately causes a repression of flowering (Fornara *et al*., 2009; Jung *et al*., 2009). The SPL family members SPL3, SPL4 and SPL5 directly induce the expression of floral promoters *LFY*, *FUL*, *AP1* (Yamaguchi *et al*., 2009). SPL9 and SPL10 regulate flowering by inducing the transcription of miR172 (Wu *et al*., 2009). *miR172* mediates the expression of AP2-like genes such as TOE1, TOE2, SCHNARCHZAPFEN (SNZ) which in turn represses *FT* (Aukerman *et al*., 2003; Jung *et al*., 2007; Zhu *et al*., 2011; Jarillo *et al*., 2011).

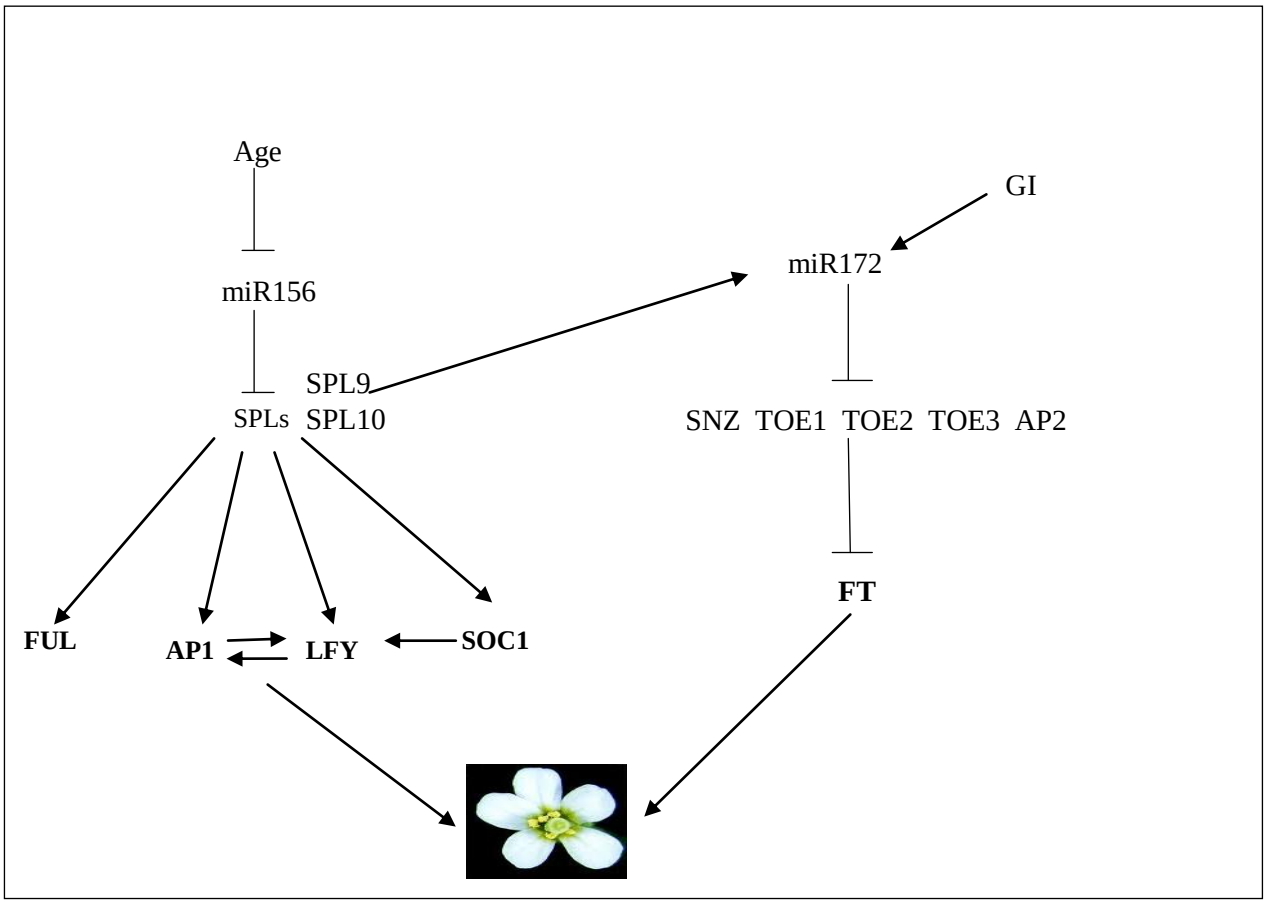


Fig 1.4 A schematic diagram of the microRNA pathway in *Arabidopsis thaliana.* Arrows indicate activation and T-bars represents inhibition. *miRNA* 156 and 172 represents microRNA 156 and 172, *SPL* represents SQUAMOSA PROMOTER BINDING PROTEIN LIKE, *GI* represents *GIGANTEA*, *SNZ* represents *SCHNARCHZAPFEN*, *TOE*1 and 2 represents *TARGET OF EAT* 1 and 2, AP1and 2 represents *APETELA* 1 and 2 and *FT* represents *FLOWERING LOCUS T*.

1.1.1.3 The Ambient Temperature and Light quality Pathway

Temperature is one of the most important factors affecting plant growth and development. Temperature also plays a vital role in the induction of flowering. Studies have shown that higher temperature triggers early flowering in *Arabidopsis* via inactivation of the *FLOWERING LOCUS M (FLM)* gene, the FLM protein represses *FT* activity (Balasubramanian *et al*., 2006).

Light quality is another important factor affecting plant growth and development. Plants can detect changes in R/FR ratio and adequately respond by altering their developmental process. To date, Five types of photoreceptors have been identified in plants they include; Zeitlupe/FKF1/LKP2 photoreceptors, blue light absorbing cryptochromes (CRYs), R/FR light absorbing phytochromes (PHYs) and phototrophins (PHOTs) and the UV photoreceptor UVR8 (Onodera *et al*., 2005). Light quality can affect *FT* expression in a CO-independent manner (Cerdan *et al*., 2003). At a low R/FR ratio, PHYTOCHROME AND FLOWERING TIME 1 (PFT1) induces the expression of *FT* and promotes flowering while at a high R/FR ratio its action is repressed by PHYB (Fig 1.5).

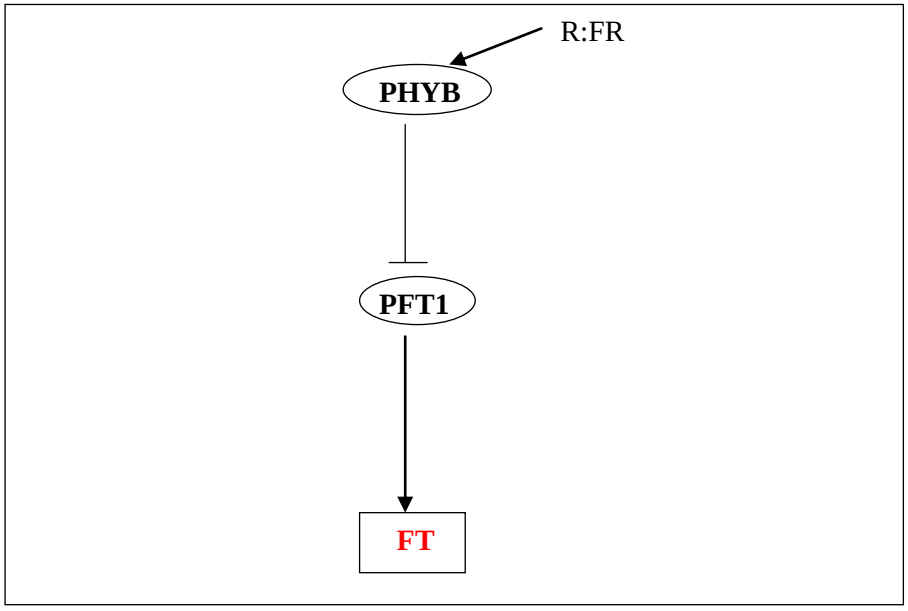


Fig 1.5 A schematic diagram of the Light quality pathway in *Arabidopsis thaliana*. Arrows indicate activation and T-bars represents inhibition. R:FR represents Red to Far Red light ratio.

1.1.1.4 The Vernalization Pathway

During extended cold periods e.g. winter, some plants adapt their growth habits to ensure maximal reproductive success (Massiah *et al.*, 2007; Kim *et al*., 2009). A vernalisation period can range from as little as four weeks to several months with temperatures ranging between 4-8^{o}C. It has been documented that some plants do require vernalization for early flowering. Various genes are known to exhibit changes in their expression levels during vernalization (Michaels *et al*., 2000). The two key genes involved in this process are *FRIGIDA* (*FRI*) and *FLC* (Fig 1.6).

FLC encodes a MADS-box domain transcription factor. It inhibits flowering by repressing *FT, FLOWERING LOCUS D* (*FD*) and *SOC1* (Boss *et al*., 2004; Searle *et al*., 2006; Kim *et al*., 2009). *FRI* inhibits flowering by inducing the expression of *FLC.* VERNALIZATION INSENSITIVE 3 (*VIN3*) which is expressed after a cold treatment represses the expression of *FLC* mRNA by causing a histone modification of *FLC* chromatin (Boss *et al*., 2004; Kim *et al*., 2009; (Sung *et al*., 2004; Geraldo *et al*., 2009; Kim *et al*., 2009).

FLC repression is subsequently maintained by intrinsic mechanisms involving other genes such *VRN1* and *VRN2* (Fig 1.6) (Gendall *et al*., 2001; Amasino 2010; Massiah *et al*., 2007).

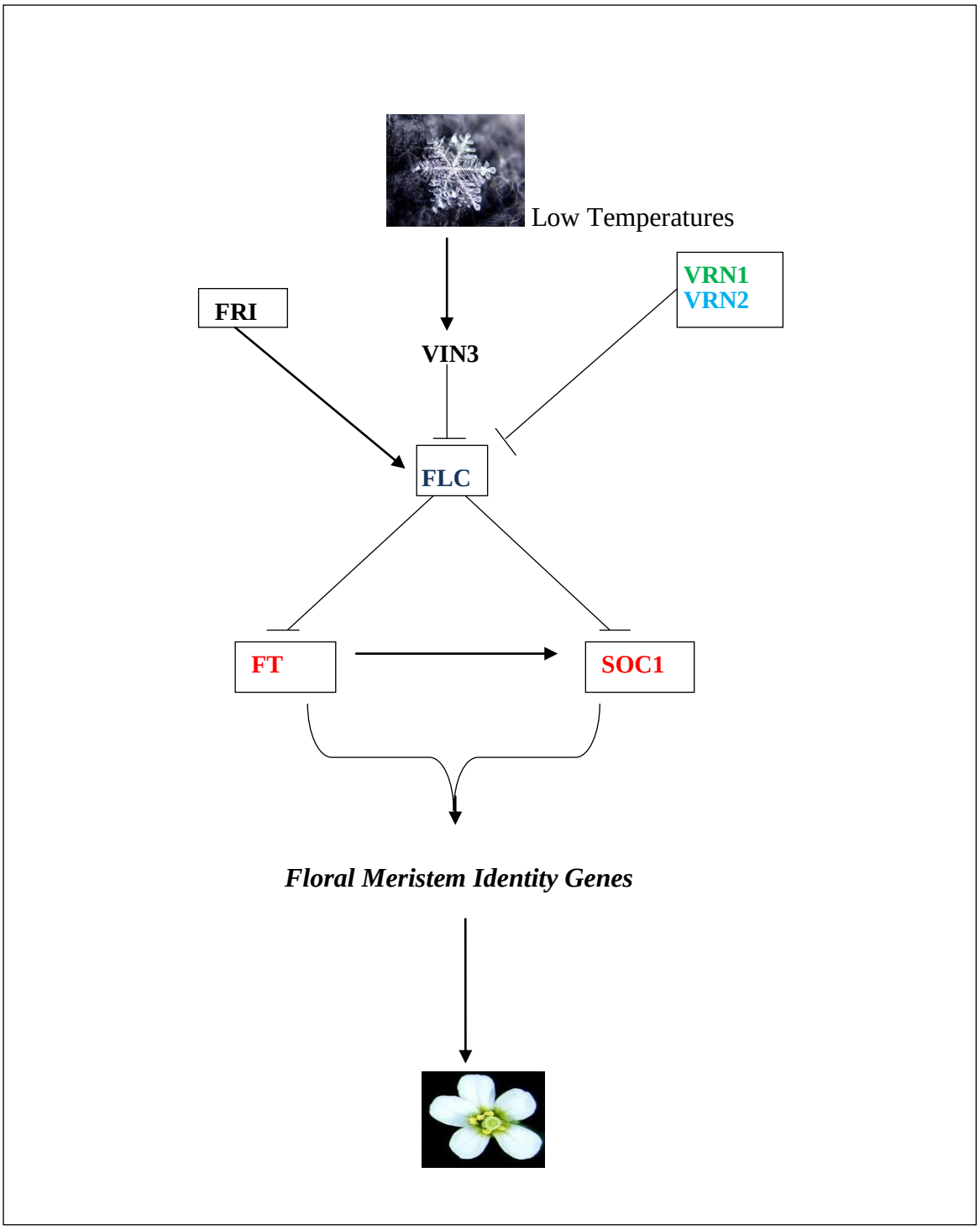


Fig 1.6 A schematic diagram of the Vernalization pathway in *Arabidopsis thaliana*. Arrows indicate activation and T-bars represents inhibition. *FRI* represents *FRIGIDA*, *VIN3* represents VERNALIZATION INSENSITIVE 3, *FLC* represents *FLOWERING LOCUS C*, *VRN*1 and 2 represents VERNALIZATION 1 and 2, *FT* represents *FLOWERING LOCUS T* and *SOC1* represents *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*.

1.1.1.5 The Gibberellin Pathway

Gibberellins are classified as plant hormones. GA promotes flowering in *Arabidopsis.* It also plays a role in cell elongation and seed germination. Previous studies showed that *Arabidopsis* mutants defective in either GA biosynthesis or signalling exhibited delayed flowering under SD (Blazquez *et al*., 1998; Wilson *et al*., 1992). Other studies showed that plants which overexpressed GA-20 oxidase flowered early in both LD and SDs (Huang *et al*., 1998; Coles *et al.*, 1999). These findings buttressed the theory of the role of GAs in inducing flowering. GAs promotes flowering via indirect activation of *SOC1* and *LFY* (Lee *et al*., 2010; Gocal *et al*., 2001) (Fig 1.7).

In addition it is also involved in the indirect repression of *miRI59* expression via down-regulation of DELLA protein levels (Fig 1.7). The repression and down regulation of *miR159*/DELLA protein levels ultimately promotes flowering through the upregulation of *LFY* and *SOC1* expression (Achard *et al*., 2004).

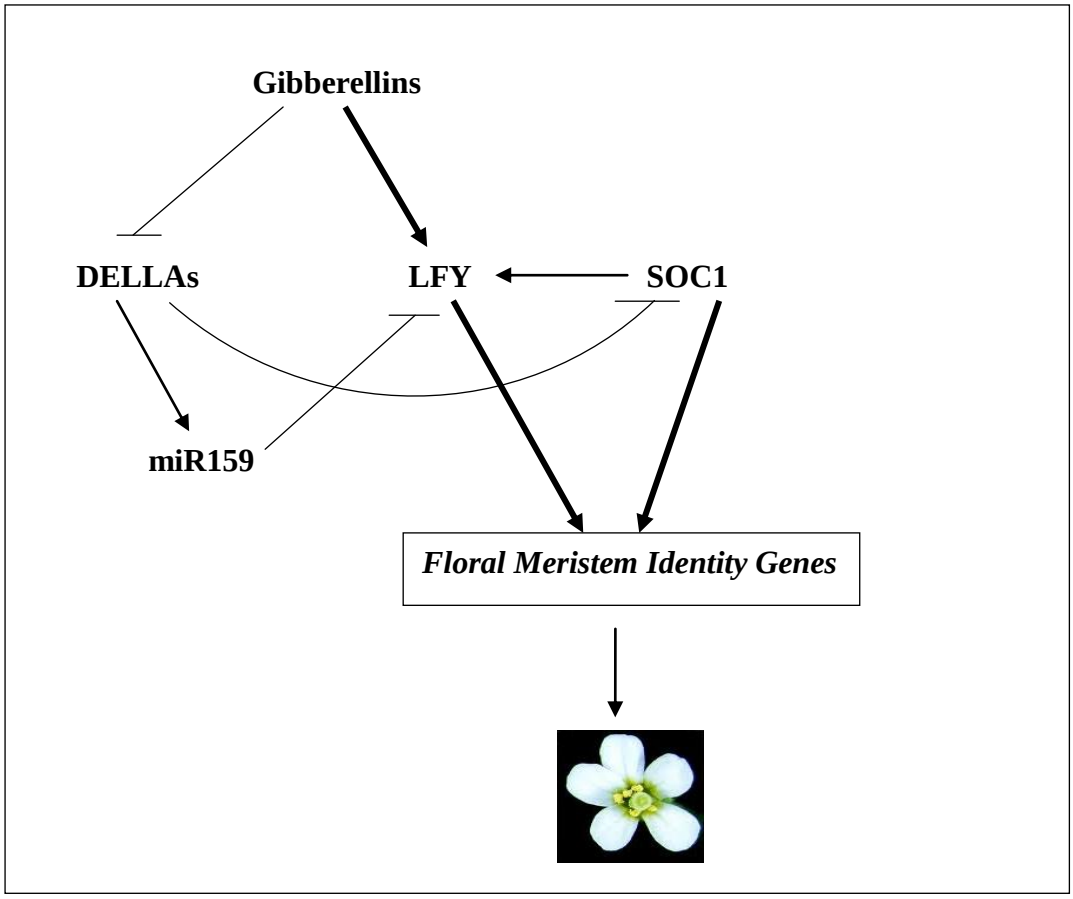


Fig 1.7 A schematic diagram of the Gibberellin pathway in *Arabidopsis thaliana.* Arrows indicate activation and T-bars represents inhibition. *LHY* represents *LEAFY, SOC1* represents *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1, miRNA* 159 represents microRNA159.

1.1.1.6 The Autonomous Pathway

The genes involved in this pathway include; *FCA*, *LUMINIDEPENDENS (LD)*, *FY*, *FPA*, *FVE*, *RELATIVE OF EARLY FLOWERING 6* (*REF6*), *FLD*, and *FLOWERING LOCUS K (FLK*) (Fig 1.8) These genes act to promote flowering by repressing the expression of the floral repressor *FLC* (Koornneef *et al*., 1991; Marquardt *et al*., 2006). *FVE* and *FLD* regulate *FLC* by chromatin modification while *FPA*, *FLK* interact with *FLC* mRNA (Simpson 2004). The autonomous pathway acts independent of environment factors (Srikanth *et al*., 2011; Massiah *et al*., 2007; Yan *et al*., 2010).

Much research has been carried out studying genes involved in the autonomous pathway for example mutants *fca*, *fy*, *fve* and *fpa* were reported to flower later than WT in both LD and SD photoperiods (Koornneef *et al*., 1998). These responses were similar to those seen in other late flowering mutants *ld* (Lee *et al*., 1994; *fld* (Sanda *et al*., 1996 and *flk* (Lim *et al*., 2004).

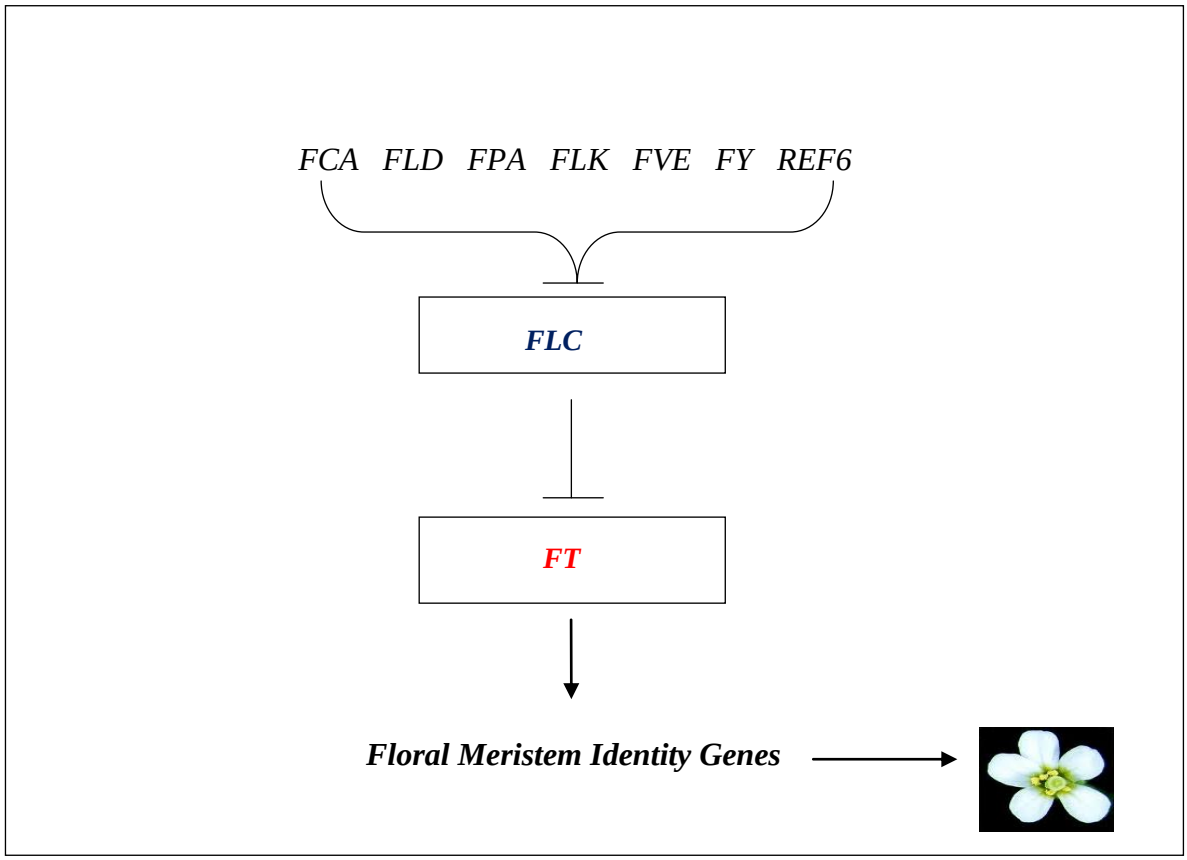


Fig 1.8 A schematic diagram of the Autonomous pathway . Arrows indicate activation and T-bars represents inhibition. *FLK* represents *FLOWERING LOCUS K*, *REF6* represents *RELATIVE OF EARLY FLOWERING 6*, *FLC* represents *FLOWERING LOCUS C* and *FT* represents *FLOWERING LOCUS T*.

1.1.1.7 Floral Integrators

The vernalization, autonomous, photoperiodic, microRNA, ambient temperature and light quality with the exception of GA pathway all converge on the floral integrator genes *FT* and *SOC1* which in turn activate the floral meristemic identity genes AP1, LFY and *FUL*. The genes induce the transition to floral induction in the developing primordia (Boss *et al*., 2004).

1.2 Florigen –The flowering time regulator *FT*

1.2.1 The Florigen hypothesis

A vast majority of plant species exhibit accelerated flowering when grown and maintained under specific photoperiod. This was demonstrated by Garner and Allard in the 1920's. They reported that late flowering tobacco strain, Maryland Mammoth exhibited early flowering when the photoperiod was reduced (Garner *et al*., 1922). Other classical experiments demonstrated that flowering was triggered in plants that were maintained under LD conditions even though the apex of the plants in which flowering occur was exposed to non-inductive SD condition (Knott 1932). These discoveries led to the conclusion that leaves were the site of signal perception.

In 1937, numerous grafting experiments were carried out by Dr Mikhali Chailakyn. He reported that flowering stimulus is initiated in the leaves under inductive photoperiodic conditions. A series of cascade of events occur which leads to the movement of the stimuli from the primary site, i.e. the leaves, to the shoot apex which ultimately causes flowering. These findings led to the florigen hypothesis. Florigen is defined as a universal chemical compound synthesised by leaves under inductive conditions which is translocated to the shoot apex where it induces flowering. Classical grafting experiments supported the florigen hypothesis. Grafting with different plant species showed that flowering was induced when a single induced leaf was grafted onto a non-induced plant (Zeevaart 1976; Corbesier *et al.*, 2006; Kobayashi *et al*., 2007).

1.2.2 The physiology of *FT* and its homologues

FT encodes a small globular protein with a molecular weight of 20kDa which is known to be structurally similar to the Raf Kinase Inhibitory Protein (RKIP) family of mammals (Kardailsky *et al*., 1999). It is also homologous to the phosphatidyl ethanolamine binding protein (PEBP) (Imaizumi *et al*., 2006). It is known that PEBPs play a vital role in signalling and also growth and differentiation in plants and animals (Hanzawa *et al*., 2005; Kardailsky *et al*., 1999). There are six genes in the PEBP gene family of *Arabidopsis*. They include *FT*, *TSF*, *ARABIDOPSIS CENTRORADIALIS HOMOLOGUE (ATC)*, *TFL1*, *BROTHER OF FT (BFT)*, *TFL I* and *MOTHER OF FT (MFT).* The six genes are sub classified into three groups. They include *TFL*-like subfamily, *FT*-like subfamily and *MFT*-like subfamily. *TFL1* and *BFT* belong to the *TFL*-like subfamily. The genes in this group are involved in flowering repression while *FT* and *TSF* belong to the *FT*-like subfamily and the genes in this group are involved in flowering induction.

TSF is structurally identical to *FT*. In 2005, Hanzawa reported that with just a single amino acid change on FT it is possible to convert it into a TFL1-like molecule (Hanzawa *et al*., 2005). Structural molecular analysis showed that key residues that confer FT or TFL1-like behaviour exist on an exposed loop of these proteins which suggests that the two proteins act through a common mechanism (Ahn *et al*., 2006). *MFT* promotes embryo growth of seeds by interacting with the GA pathway (Xi *et al*., 2010). It also induces early flowering as reported by Dr Yoo, *Arabidopsis* lines that had over expressed *MFT* exhibited early flowering phenotype compared to WT plants (Yoo *et al*., 2004).

1.2.3 Conservation of *Arabidopsis* flowering genes in crop species

The knowledge of *FT* in *Arabidopsis* has pioneered the discovery and understanding of other *FT* orthologues in crop species. Comparative genome analysis with rice sequence revealed that a majority of the *Arabidopsis* key flowering genes were conserved (Izawa *et al*., 2003). Orthologues of *FT* (*Hd3a*), *GI* (*OsGI*) and *CO* (*HD1*) have been identified in rice a SD plant. In rice, *OsGI* promotes the expression of *HD1* as seen in *Arabidopsis* with *GI* and *CO*.

HD1 plays a crucial role in mediating the photoperiodic signal. It activates *Hd3a* expression in rice under SDs whereas in LDs it inhibits *Hd3a* (Izawa *et al.*, 2002; Hayama *et al*., 2003; Kojima *et al*., 2002).

In Tomato, a DNP, flowering is not affected by photoperiod (Lifschitz *et al*., 2006). However tomato plants exhibit processes that are regulated by the circadian clock, although floral induction is not one of these (Jarillo *et al*., 2008). The tomato *FT* orthologue *SINGLE FLOWER TRUSS* (*SFT*) has been reported to play a role in the promotion of flowering (Lifschitz *et al*., 2006). The *SFT* gene has also been proposed to have a role in floral induction through the autonomous pathway (Molinero-Rosales *et al*., 1999). Other tomato *FT*-like genes include *SELF PRUNING* (*SP), SP2I, SP3D, SP5G, SP6A* and *SP9D*(Carmel-Goren *et al*., 2003). These genes control the regularity of the vegetative –reproductive switch in tomato (Carmel-Goren *et al*., 2003). Over expression of *SP* resulted in an increased number of leaves between inflorescences and an increased leafiness of the inflorescence shoot itself (Pnueli *et al*., 1998). Divergence expression studies in the *SELF PRUNING* (*SP*) family indicated various expression levels in vegetative and reproductive organs (Carmel-Goren *et al*., 2003), e.g. *SP3D* was expressed mainly in floral organs while *SP5G* was expressed only in the cotyledon and leaves. *SP2I* was quite unique because it was discovered to be expressed in all the vegetative and reproductive organs. Perhaps the *SP2I* gene has a role in both the vegetative and reproductive phase transition in tomato.

In barley, a LD plant, 5 highly conserved genes homologous to *FT* have been identified. *HVFT1* was discovered to be barley *FT*-like gene involved in flowering regulation (Faure *et al*., 2007). Two *CO*(*HvCOI* and *HvCO2*) and a barley *GI* (*HvGI*) gene was also reported (Dunford *et al*., 2005). *PHOTOPERIOD-HI* (*Ppd-HI*) which is a pseudo-response regulator has been reported to be the major component of flowering in LD photoperiods in barley (Decousset *et al*., 2000). *Ppd-h1* mutants exhibit reduced photoperiod which was as a result of an altered circadian expression of *CO* and a reduced expression of *FT* (Turner *et al*., 2005).

In the potato *Solanum tuberosum ssp. andigena* which is qualitative SD plant homologues of *CO* (*StCOL3*), *GI* (*StGI*) and *FT* (*StFT*) have been identified. These homologues have been suggested to have a key role in tuberization control. In 2006, Dr Rodriguez-Falcon proposed that the control of tuberization by *StCOL3* is mediated by the regulation of *FT* activity through the PHYB-dependent mechanism of regulation similar to the mechanism reported in

rice (Rodriguez-Falcon *et al*., 2006). Under LDs PHYB interacts with *StCOL3* causing a repression in the expression of *StFT* whereas in SDs, *StCOL3* induces the expression of *StFT*. Phytochrome B had previously been shown to have a role in the photoperiodic control of tuberisation (Jackson *et al*., 1996). In mutant *phyB* lines in which PHYB activity was repressed, the plants tuberized in LDs while WT plants did not tuberize. Potentially of interest to this project is the work carried out on tuberisation in potato. We will investigate the role that *FT* plays in the induction of tuberization in potato.

In 2011, *FT*-like paralogues; *StSP6A* and *StSP3D* were reported to have been discovered in potato. These genes had key roles in tuberization and floral induction respectively (Navarro *et al*., 2011). Transgenic Andigena lines that over-expressed *StSP6Aox* tuberized under non-inductive LDs while lines in which the target gene was silenced did not tuberize under the same conditions. Andigena lines in which the *StSP3D* was down-regulated exhibited a late flowering response. Flowering was reported to be completely suppressed in lines that had strong silence levels. This showed that different FT-like genes had different roles in potato.

1.2.4 The Role of FT protein in long-distance transport and flowering induction

Over the years much work has been carried out to elucidate the transport of FT protein and its role in floral induction. The subject of FT protein transportation in plants proved to be a controversial topic. FT protein alone was initially thought to be able to move from the vasculature to the apex and induce flowering. In 2005, Huang *et al* reported that *FT* mRNA was a mobile signal in *Arabidopsis*. They expressed *FT* fused with a *GUS* reporter gene driven by a heat shock-inducible promoter. Elevated levels of *FT* transcripts were detected in the vasculature after induction. In addition the transcripts were also detected in the shoot apex several hours later. This result indicated that the *FT* mRNA could move from an induced leaf to the apex of the plant. The paper was retracted due to the fact that the experiment could not be reproduced (Bohlenius *et al*., 2007). Jaeger *et al*.,2007 reported that FT protein could travel from the vasculature to its site of action at the SAM where it interacts with *FD* to initiate floral induction in the plant. They demonstrated this by generating immobile FT proteins. A nuclear localisation signal (NLS) was attached to the FT protein to inhibit its movement out of the cell. When the fusion protein was expressed in *ft* mutants driven by a vasculature specific promoter, there was no flowering but when the

NLS tag was removed flowering occurred. This led to the conclusion that FT protein was a mobile signal that is capable of long distance transportation. This finding was consistent with other findings reported at that time. For instance, in *Brassica napus*, FT protein was identified in the soluble fraction of sieve-tube exudates of *brassica* phloem sap (Giavalisco *et al*., 2006).

Although it is widely acknowledged that FT protein is a mobile floral stimulus which could move from the vasculature to the SAM, the question of whether *FT* mRNA could also move is still under debate. In recent years, Li *et al* (2009) demonstrated that *FT* mRNA was capable of long distance movement. They used a RNA mobility assay based on movement defective viruses; *Potato Virus X* and *Turnip crinkle virus*, and mutant and WT *Arabidopsis FT* genes were independently cloned into these movement defective viruses. The mutant *FT (*m*FT)* clone contained a non-translatable version of *FT* in which the start codon (ATG) had been replaced with a stop codon (TAG) (Fig 1.9). They reported that the non- translatable *FT* mRNA (mutant) could move throughout *Nicotina benthamina*, *Maryland Mammoth* and *ft* mutant *Arabidopsis* plants and thus the movement was independent of FT protein (Li *et al*., 2009).

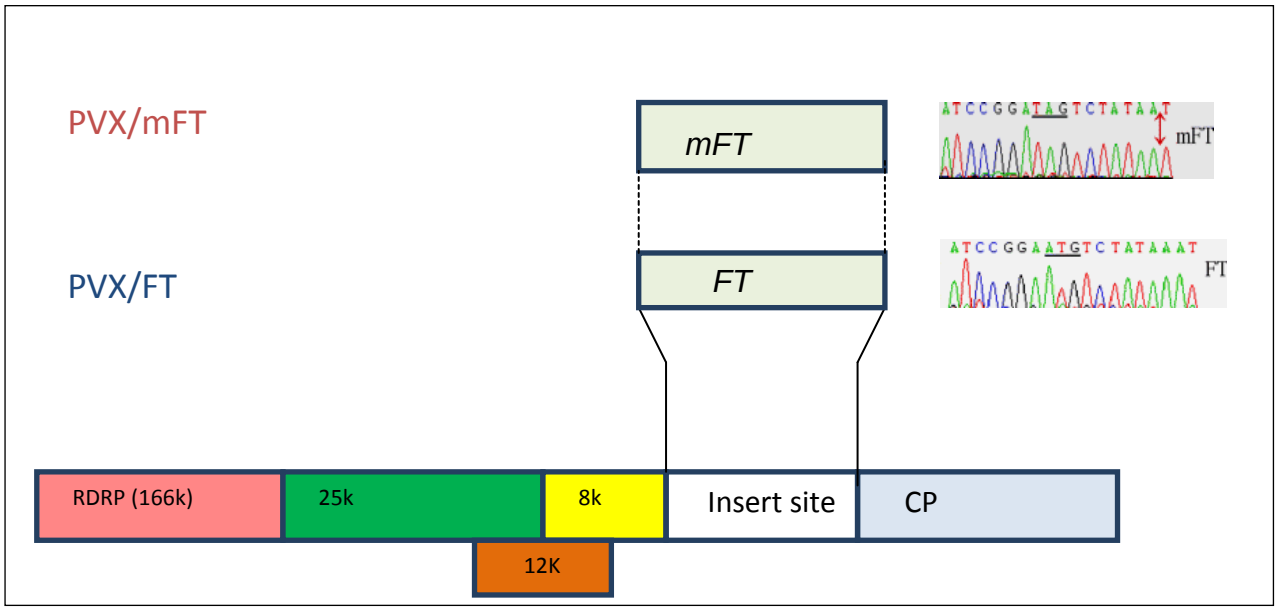


Figure 1.9. A schematic representation of the constructs used for experiment. The PVX/mFT construct contains a non-translatable mutant version of *FT* in which the start codon (ATG) has been replaced with a stop codon (TAG), and there is also an additional adenine to thymine mutation at codon 4.

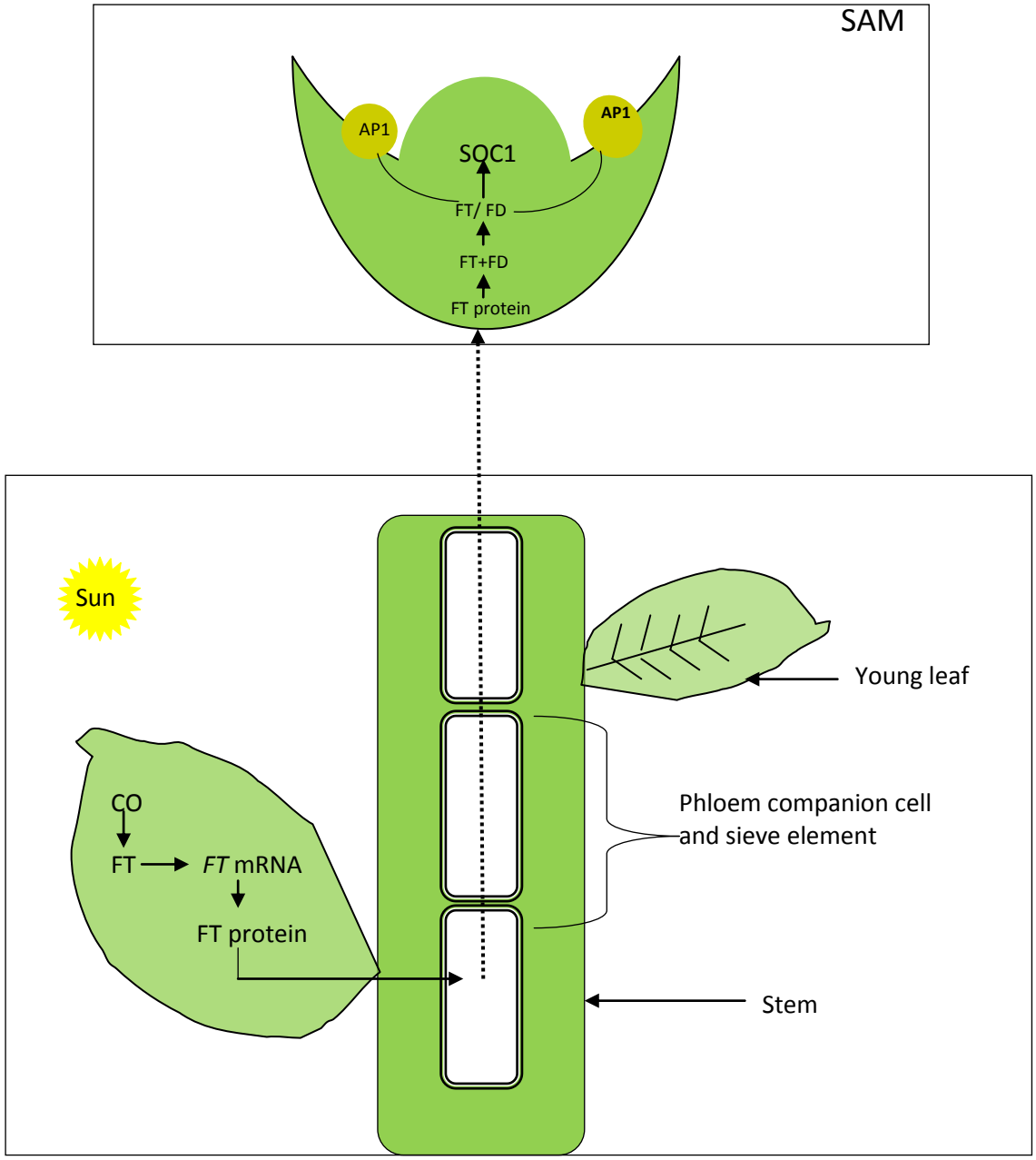


Figure 1.10 The role *FT* in long-distance transport and flowering induction. Light drives the rhythm of *CO* expression. CO activates the *FT* gene. *FT* mRNA / FT protein move from the leaf to the shoot apical meristem (SAM) through the phloem's sieve plates and sieve elements. At the SAM the FT protein interacts with the FD bZIP transcription factor forming a complex which results in the direct upregulation of *SUPPRESSOR OF CONSTANS 1* (*SOC1*) mRNA. SOC1 in turn forms a complex with AGAMOUS-LIKE 24 (AGL24) which translocates to the nucleus where it binds the *LEAFY* (LFY) promoter to induce *LFY* expression which ultimately induces the development of floral primordia**.** The FT/FD complex could also induce the expression of floral meristem identity genes such as *APETALA1*(*AP1*) as indicated above.

1.3 Utilization of Plant virus-based toolbox to investigate protein function and RNA movement

1.3.1 Potato Virus X (PVX) and PVX-based viral expression system

In recent years, the PVX vector has become a tool of interest to plant biologists because of its relative stability, ease of host infection and genomic manipulation, high titers in infected plants and its mechanical transmissibility to a number of Solanaceous hosts e.g. tobacco, tomato, potato, eggplant, pepper etc. (Angell *et al*., 1997).

PVX is a potexvirus. It is also a filamentous rod-shaped virus which contains a single plus sense RNA molecule. The 5' end of PVX RNA has an m^7GpppG cap and the 3' end has a polyadenylated tail. The PVX genome encodes 5 open reading frames that include RNA-dependent RNA polymerase (RdRp), Triple gene block (TGB); TGBp1, TGBp2 and TGBp3, and coat protein (CP) (Huisman *et al*., 1988). The ORFs holds the key factors responsible for virus survival and mobility. The RNA-dependent RNA polymerase (RdRp) gene is located at the 5'end of the PVX genome. The RdRp is solely involved in interacting with host factors to promote PVX replication (Angell *et al.*, 1997). The three encoded TGB proteins TGBp1 (25kDa), TGBp2 (12kDa), and TGBp3 (8kDa) have roles in virus transport. They are known to be conserved among members of the *Potexvirus*, *Furovirus*, *Hordeivirus* and *Carlavirus* genera (Angell *et al*., 1997; Wodnarfilipowicz *et al*., 1980). TGBp is also able to move intercellularly through expanding plasmodesmata (Kalinina *et al*., 1996; Krishnamurthy *et al.*, 2002). Coat protein (CP) is involved in cell to cell movement and systemic spread in host and it is also an important structural protein for assembling virus particles (Chapman *et al*., 1992; Scholthof *et al*., 1996; Angell *et al*., 1999).

The PVX vector used in this project was derived from pPC2S. The modified viral cDNA was positioned 3' to the T7 RNA polymerase promoter (Baulcombe *et al*., 1995). The cloning sites within the PVX plasmid are between the *Cla I* and *Sal I* sites. There is also an *EcoRV* site that can be used for cloning of blunt ended sequences. After cloning the gene of interest into the plasmid, the recombinant plasmid is linearised before *in vitro* transcription is carried out to generate the infections with RNA. *Arabidopsis FT* and mutant *FT* were cloned into the PVX plasmid. It was shown that the overexpression of *Arabidposis FT* from PVX in tobacco induces early flowering under non inductive photoperiods (Li *et al*., 2009). The mutant *FT*

mRNA and *Arabidopsis FT* were also both shown to move from the leaf tissue to the apex providing evidence that *FT* RNA is involved in long distance movement (Li *et al*., 2009). The mutant *FT* construct possessed a premature stop codon at the start of the sequence, hence a non translatable *FT* mRNA is produced and no FT protein is made.

1.4 Project aims

The principal aim of this project was to utilize viral vectors expressing *FT* and *FT* orthologues to further our understanding of floral induction and to investigate their commercial potential in plant breeding programmes. The specific objectives were:

- Investigate whether viral expression of *FT* can induce rapid flowering in a range of crops including Brassica, tomato, tobacco and potato.

- Investigate whether the viral expression of *FT* can induce tuberisation in potato.

- Address issues related to the potential use of PVX/FT in commercial breeding

Chapter 2

General Materials and Methods

Chapter 2: General Material and Methods

2.1 General materials

2.1.1 Plant materials

Plant species used in this project were; SD-requiring potato (*S. Tuberosum* L. subsp. *Andigena* 7540)(Jackson *et al*., 1996), SD Maryland Mammoth tobacco (*Nicotiana tabacum)*(Garner *et al*., 1922), *day- neutral* tobacco (*Nicotiana benthamiana), tomato (Lycopersicon esculentum*) Ailsa Craig kindly provided by Dr Andrew Thompson (Warwick) and broccoli (*Brassica oleracea var.italica*) kindly provided by Prof. Brian Thomas (Warwick). The potato plants were obtained by subculturing plants into fresh 2xMS media and then maintained in a sterile environment. Mature potato plants were transplanted into soil for subsequent experiments. Seeds of tomato, tobacco and broccoli were sown into F2S compost (Levingtons). Young seedlings were transferred into separate pots containing M2 compost (Levingtons) and maintained in an insect free containment glasshouse under LD condition.

2.1.2 Bacterial material and media

Bacterial strains used included electrocompetent *E.coli* EC100 (Cambio Ltd., Cat No. EC10005) and *Agrobacterium tumefaciens* (AGC58PGV3101). *E.coli* EC100 strain; F^- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ^- rpsL (Str^R) nupG) was used in the generation of recombinant virus constructs and *Agrobacterium tumefaciens* (AGC58PGV3101) strain; pMP90RK ΔT-DNA- pTiC58+ *vir* was used in agroinfiltration assay. LB, SOB and SOC media were used for bacterial growth in this project. For the preparing of 200ml LB medium, 5g of LB broth powder, Miller (Merck) was dissolved in double distilled water in a final volume of 200ml and autoclaved. In the case of LB Agar plates, 3g of Agar powder (Merck) was added to 200ml of LB and autoclaved. For the preparation of 200ml of SOB medium, 1g of Yeast extact (Difco), 4g of Bacto-Tryptone (Difco), 1.10mg of NaCl (Sigma), and 560ng of KCl (Sigma) were dissolved in double distilled water to a volume of 200ml and autoclaved. For the preparing of 200ml of SOC medium, 4g of bacto-Tryptone and 1g of bacto-yeast extract were dissolved in 190ml double distilled water. The resulting mixture was then autoclaved and cooled down.

After cooling, separately autoclaved 400µl of 5M NaCl, 2ml of 1M $MgSO_4$, 4ml of 1M Glucose and 500µl of 1M KCl were added in a sterile environment to make up the volume to 200ml.

2.1.3 Plant virus –based vectors

RNA mobility assay (RMA) vector used in this project was based on *Potato Virus X* (PVX Fig 2.1)(van Wezel *et al*., 2001) which was kindly provided by Prof Yiguo Hong (Warwick).

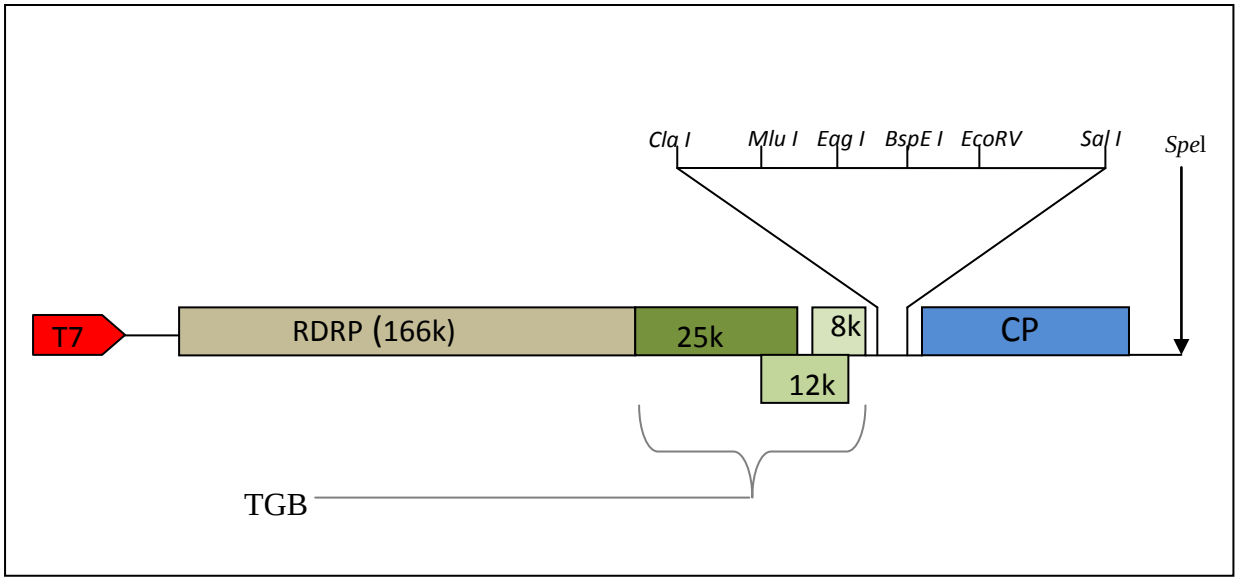


Figure 2.1. A Schematic representation of the plasmid vector (PVX) used for cloning. The RNA dependent RNA polymerase (RDRP) (166K) is involved in promoting PVX replication while the Triple gene block (TGB) subunits (25K, 12K and 8K) are involved in PVX transportation. The restriction sites within PVX include *ClaI, MluI, EagI, BspEI, EcoRV* and *SalI*. The PVX vector is a modified version of pP2C2S (Baulcombe *et al*., 1995). The modified vector contains a T7 promoter and a unique *Spe*l site for plasmid linearization.

2.2 General methods

2.2.1 Plant inoculation with Tissue Sap

Young SD *N.tabacum* Maryland Mammoth, *day- neutral* tobacco (*Nicotiana Benthamiana),* tomato *(Lycopersicon esculentum*) Ailsa Craig, broccoli (*Brassica oleracea var.italica*) and SD-requiring potato (*S. tuberosum* L. subsp. *Andigena* 7540) plants were inoculated at the 5-6 leaf stage. Inoculation was carried out on 5 plants per construct. The plant's leaves were initially dusted with carborundum powder in order to cause abrasion on the leaf surface (Fisher Scientific) and then inoculated with sap from PVX/FT, PVX/FTC4, PVX/FT-HIS, PVX/FT-FLAG, PVX/mFT infected tobacco plants. Two young leaves were inoculated for each plant subject. After inoculation, plants were maintained in an insect-free containment glasshouse in a LD (16hr) photoperiod.

2.2.2 Plant growing conditions

Seeds of *Nicotiana tabacum* Maryland Mammoth *CP*-transgenic line 1C and WT *Nicotiana tabacum*, tomato *(Lycopersicon esculentum*) Ailsa Craig, broccoli (*Brassica oleracea var.italica*) and SD-requiring potato (*S. tuberosum* L. subsp. *Andigena* 7540) were sown in F2S compost (Levingtons). After the seeds had germinated, seedlings were transplanted into new separate pots containing M2 compost (Levingtons) and grown at 25^{o}C under LD condition.

2.2.3 Tissue culturing and Shoot induction media

For the preparation of 1L of tissue culture media, 8.1g Murashige and Skoog (MS) powder (Duchefa Biochemie) was dissolved in 800ml of sterile water. 40g of sucrose (Sigma) was also added to the mixture. pH was adjusted to 5.8 with 1M NaOH or 1M HCl as necessary while stirring the mixture. Sterile water was added to make up a total volume of 1L. 7g of agar (Sigma) was added to the mixture and autoclaved. Molten MS media was aseptically poured into tissue culture pots and allowed to solidify. The MS media was used for subsequent tissue culturing. For the preparation of Shoot induction media (MG), MS media was prepared as mentioned above with the exception of sucrose which was replaced with 1.6% glucose (Sigma). After autoclaving and keeping the mixture warm, 1mg/l of

Benzylamino purine (BAP), 0.2mg/l alpha-naphthalenacetic acid (NAA) (Sigma) dissolved in DMSO, 100mg/l ampicillin and 50mg/l spectinomycin were added into the mixture. Molten MG media was aseptically poured into tissue culture pots and allowed to solidify. The MG media was used for subsequent tissue culturing.

2.2.4 Tissue culture of potato and tobacco plants

Solid 2xMS media was melted in the microwave and aseptically poured into sterile pots. Working in a flow hood and with the aid of sterile forceps and scalpels plant tissue material was excised from parent plant and subcultured into new properly labelled media pots. The plants were nurtured and allowed to mature. This took between 4-5 weeks. When the plants were mature enough they were transplanted into M2 compost (Levingtons).

2.2.5 Seed Sowing (Filter paper and Soil)

In the experiment investigating whether the virally expressed *FT* and *CP* could move into the germline of PVX/FT inoculated plants, seeds were harvested from the seed pods of PVX/FT inoculated *N. tabacum and L. esculentum* plants and sterilised in 10% bleach (domestos). The seeds were air dried and sown on wet filter paper in sterile petri-dishes (Thermo Fisher). Germination occurred between 2-3 weeks. Young seedlings were harvested after which RNA extraction was carried out with the aid of an RNAeasy Kit (QIAGEN) as suggested by manufacturer's protocol.

2.2.6 High fidelity KOD-PCR

High fidelity KOD DNA polymerase (Merck Chemicals, Cat No 71086) together with gene specific primers (see Appendix) was used in the amplification of DNA fragments to produce different gene expression vectors. A standard KOD-PCR was set up in 20µl containing 2µl of 1X KOD hot start DNA Polymerase reaction buffer, 1.5µl 2mM $MgSO_4$, 2µl template DNA (approx. 10-100ng), 1µl 0.2mM dNTPs, 0.4µl 0.5µM of each forward and reverse primers and SDW to make up 20µl. PCR was carried out at an initial denaturation at

94^{o}C for 2mins, followed by 30cycles (if not specified otherwise) of denaturation at 94^{o}C for 15 sec, annealing (specific primer temperature) for 30sec, and extension at 72^{o}C for 1min per kb of expected product. A further 10 minutes of extension at 72^{o}C was carried out at the end of the cycles. Lists of primer sequences and target genes are shown in the appendix Table 1. PCR products were analysed by agarose gel electrophoresis. 1kb plus DNA ladder (Invitrogen Ltd., Cat No 107787) was run alongside the samples used to assess the fragment sizes of PCR products. 2 µl Orange G (Sigma-Aldrich, Cat No 03756) loading buffer and 6µl of samples were loaded on 1% agarose gel (Invitrogen Ltd cat No 15510,USA) and electrophoresed in 1 X TAE buffer containing 40mM Tris-acetate and 1mM of EDTA (Fisher BioReagents) at 150V for 40mins. Agarose gels were stained with GelRed™ Nucleic Acid gel stain (Biotium). Gel Images and records were taken using a G:BOX gel documentation system (Syngene, UK).

2.2.7 Purification of PCR products from gels

Nucleic acids containing bands were excised from the gel and products were isolated using a QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28704) following the manufacturer's suggested guidelines. The purified products were eluted in 20µl of SDW.

2.2.8 Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from leaf and plant tissue material (see 2.2.22)**.** The extracted RNA was dissolved with RNAse–free water (Promega). cDNA was synthesised by setting up the following: reaction volume was set up in 12 µl which contained 1 µl of random primers (Invitrogen), 1µl of 10mM dNTP mixture and 50ng of total RNA treated with DNase (Promega). Nuclease-free water (Promega) was added to make up the final total volume to 12 µl. Using a thermocycler, the mixture was heated to 65^{o}C for 5 minutes after which the mixture was quickly chilled on ice for 5 seconds. 4µl of 5x First-Strand buffer (250mM Tris-HCl, pH 8.3 at 25^{o}C; 15mM $MgCl_2$; 375mM KCl) and 2µl of 0.1M DTT was added to the mixture and incubated at 25^{o}C for 2 minutes. After the incubation time had elapsed 1 µl of

Superscript® II reverse transcriptase (Invitrogen) was added to the reaction mixture and incubated at 25°C for 10 minutes, then at 42°C for 50 minutes. Reaction mixture was inactivated at 70°C for 15 minutes. Synthesised cDNA was used as a template for subsequent PCR reaction. Standard PCR was carried out for 30 cycles (see 2.2.6). The primers PP82 and PP356, TCPF and TCPR, EFNBF and EFNBR were used to detect *Arabidopsis FT*, m*FT*, CP, PVX cDNA and Elongation factor 1-alpha (housekeeping gene). List of all primers used is shown in (Table 1).

2.2.9 Digestion of KOD –PCR products with restriction endonucleases

Purified KOD-PCR products were digested with *Eag*I and *Sal*I restriction endonucleases (New England Biolabs). The digestion reaction was typically set up in 50µl containing 30 units of each restriction enzymes, 5µl of 10 x BSA (10mg/ml), 5µl 10 x NEBuffer (see Appendix 11), 28µl of purified PCR products. A final volume of 50µl was acquired by adding sterile distilled water. The digestion reaction was carried out at 37°C for 3 hours. After the duration had elapsed, the digested DNA fragments were purified using QIAquick PCR purification kit (Qiagen) and DNA was eluted with 25µl EB buffer (Qiagen).

2.2.10 Preparation of cloning vectors

PVX vectors were digested with *Eag*I and *Sal*I restriction endonucleases (New England Biolabs). The digestion reaction was typically set up in 40µl containing 20 units of each restriction endonucleases, 4µl of 10 x BSA (10mg/ml), 4µl of 10 x NEBuffer (see Appendix 11), 1 µg of vector (miniprep plasmid DNA). A final volume of 40µl was acquired by adding sterile distilled water. The digestion reaction was carried out at 37°C for 3 hours. After the duration had elapsed an equal volume of Phenol:chloroform:isoamyl alcohol 25:24:1 (40µl) was added to the digestion mixture and then gently vortexed for 20-30seconds. The mixture was then centrifuged for 3 minutes at 15000 rpm and then 40µl of upper phase was transferred to a new 1.5ml Eppendorf tube. An equal volume of Chloroform:isoamyl

alcohol:24:1 (Sigma) (40µl) was added then the tube was gently vortexed for 20-30seconds and centrifuged for 3 minutes at 15000 rpm. The supernatant was immediately transferred to a fresh 1.5ml tube. 8µl of 3M sodium acetate and 100µl of 100% ethanol was added to the tube. The resulting mixture was temporarily stored at -20^{o}C for an hour and centrifuged at 15000 rpm at 4^{o}C for 18 minutes. Following washing in 70% Ethanol, the pellet was air-dried and dissolved in 40µl of water. The final concentration of linearised DNA vectors was 25ng/ µl.

2.2.11 Ligation reaction preparation

Ligation reaction was carried out in 30µl containing 3µl of 10 x T4 Ligase reaction buffer (500mM Tris-HCl; 100mM $MgCl_2$; 10mM ATP; 100mM DTT; pH 7.5 at 25^{o}C), 1 µl of T4 DNA ligase (New England Biolabs), 5µl of vector (10ng/µl) and 21µl of KOD –PCR products (approx. 2.5 µg DNA) digested with *Eag*I and *Sal*I restriction endonucleases (New England Biolabs). Ligation was typically carried out at room temperature overnight. Purification of ligation mixture was carried out using Phenol:chloroform extraction (see 2.2.19). Pellet was dissolved in 20µl sterile distilled water. The purified recombinant plasmid was then used for transformation.

2.2.12 Transformation of *E.coli* (EC100) by electroporation

Recombinant plasmids were transformed into *E.coli* (EC100) competent cells. 2µl of plasmid DNA(2.5 µg) was added to a 0.5ml tube containing 10µl of electro-competent cells and gently mixed by resuspension with a pipette at room temperature. The mixture was transferred into an electroporation cuvette (1mm Gap width, BioRad) and placed into Electroporator (BioRad Gene Pulser™) for electrical pulsing applying 1.5KV, 25µF capacitance and 200 Ω. After electrical pulse had been applied, 0.5ml of room temperature SOC media was added to the transformation mixture and incubated for 1 hour at 37^{o}C plated unto LB plates.

2.2.13 DNA transformation of *Agrobacterium tumefaciens*

Recombinant Binary vector pB2GW7/CP was transformed into *A. tumefaciens* competent cells by electroporation. 4µl of binary vector was added to a 0.5ml tube containing 40µl of *A. tumefaciens* competent cells **(**AGC58PGV3101) and gently mixed by resuspension with a pipette at room temperature. The mixture was transferred into an electroporation cuvette (1mm Gap width, BioRad) and gently placed into Electroporator (BioRad Gene Pulser™) for electrical pulsing applying 1.8KV for 5ms. After electrical pulse had been applied, 0.5ml of room temperature SOC media was added to the transformation mixture and plated out unto LB and incubated overnight at 28^{o}C. 25µg/ml Gentamicin (GENT), 100µg/ml Spectinomycin (SPEC) and 50µg/ml Rifampicin (RIF) (Sigma) was used for selection of transformants.

2.2.14 Tobacco Leaf disc transformation

A.tumefaciens (AGC58PGV3101) harbouring the pB2GW7/CP plasmid was grown overnight in a 10ml LB culture containing 25µg/ml Gentamicin, 100µg/ml Spectinomycin and 50µg/ml Rifampicin. The bacteria culture was centrifuged at 6500rpm for 4 minutes and then resuspended in antibiotic – free LB broth. Small pieces of leaf disc were cut from young WT *N. tabacum* plants and immersed into the bacterial suspension containing 3ml *of A. tumefaciens* and 7ml MS liquid (see 2.2.3). The leaf disc was left in the bacteria suspension for 10 minutes with occasional gentle shakes every 2 minutes. After this, the leaf discs were placed in solid 2MS media plates and incubated in the dark for 2 days at 28^{o}C. After incubation, the leaves were placed on a MG media (see 2.2.3) with periodical media replacement every 7-10 days. Shoot tissue (1-2 cm long) were excised from calli and placed in fresh MG media that contained the appropriate antibiotics (Fig 2.2B). Young transformed *N. tabacum* plants were then transferred to M2 soil (Levingtons) when they had developed roots (Fig 2.2E).

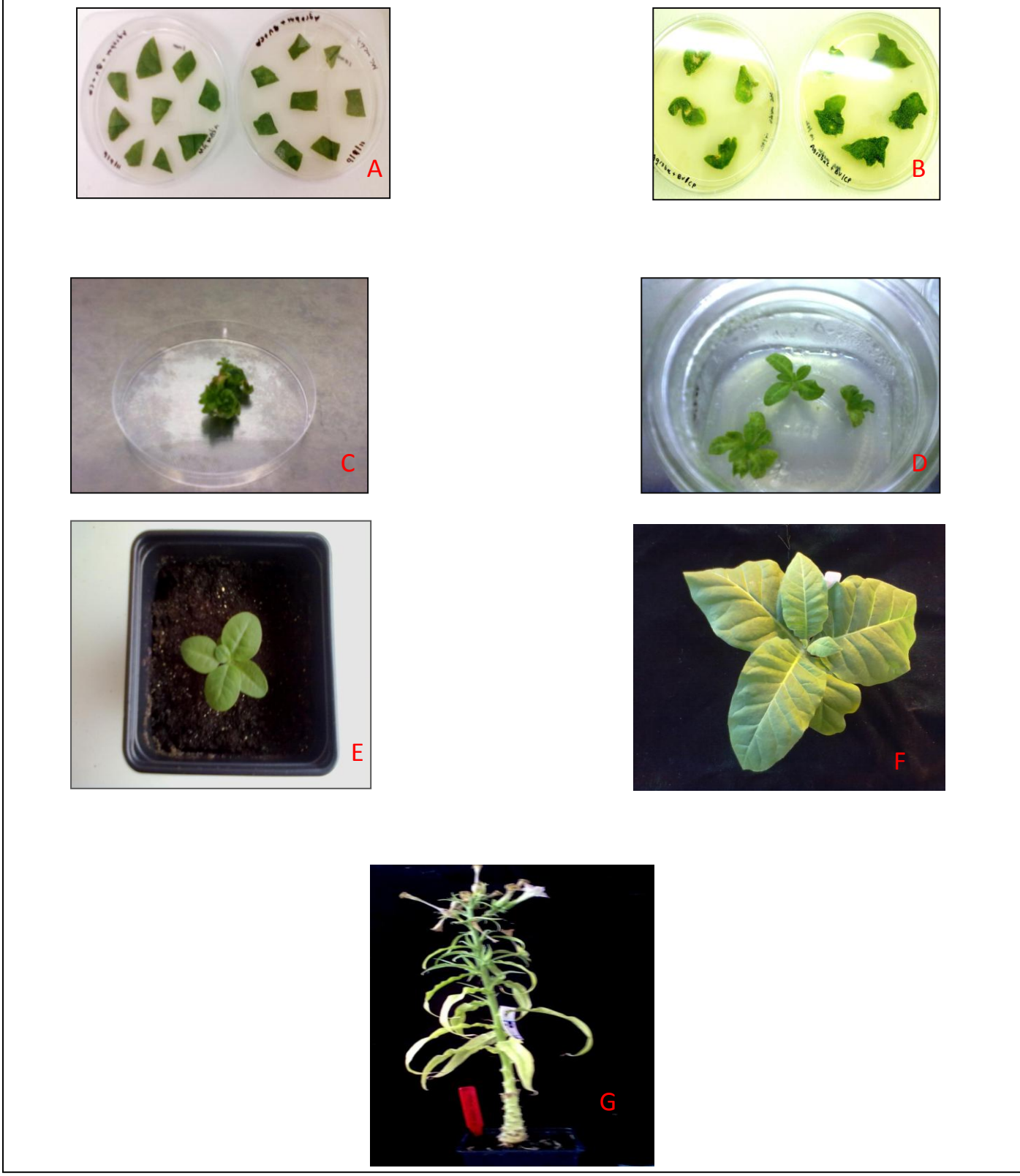


Figure 2.2 Tobacco leaf disc transformation. A shows CP recombinant *Agrobacterium tumefaciens* transformed *N.tabacum* leaf discs in shoot induction media; **B** shows calli development on transformed leaf discs in selective medium containing spectinomycin and ampicillin antibiotics; **C** shows young shoots sprouting from callus tissue; **D** shows young shoot tissue growing in MG media containing spectinomycin and ampicillin antibiotics; **E** and **F** shows a young transgenic plant growing in soil at different developmental stages; **G** shows a mature CP transgenic *N tabacum* plant in glasshouse.

2.2.15 Colony PCR screening

Individual colonies were picked and resuspended in 30 µl sterile distilled water. 1 µl of the resuspended colony was added to a preprepared PCR mixture. Standard PCR reaction (see section 2.2.6) was carried out using gene specific primers (see section appendix 1). The primers used for screening of colonies transformed with PVX-based constructs were PP82, PP356 and gene specific reverse primers (see appendix 1).

2.2.16 Extraction of plasmid DNA

Positively transformed colonies were used to inoculate 10ml of LB broth containing 100µg/ml ampicillin. The culture was propagated in a rotating incubator at 37^{o}C overnight. The culture was then centrifuged at 4000rpm (5810R, Eppendorf) for 20 minutes. Plasmid DNA was extracted using Qiaprep miniprep kit (Qiagen). DNA was eluted with 200 µl EB buffer (10mM Tris-HCl, pH 8.5).

2.2.17 Quantification of RNA and DNA samples

NanoDrop™ ND-100 spectrophotometer (Thermo Scientific) was used to measure the concentration of both DNA and RNA samples. 4µl of the RNA or DNA sample was loaded on the spectrophotometer's pedestal. The 260nm/230nm and 260nm/280nm ratios were measured. Typically pure DNA and RNA nucleic acids recorded 260/280 ratio of ~ 1.8 or a 260/280 ratio of ~ 2.0, respectively.

2.2.18 DNA sequencing

The Dye® terminator V.3.1 cycle sequencing kit (Applied Biosystems) was used to sequence DNA products. The manufacturer's protocol was followed as instructed. Template DNA was added into an Eppendorf tube containing 2µl of Big Dye and 3.2µM primer concentrations for both forward and reverse in a final volume of 10µl made with sterile distilled water.

The sequencing reaction was carried out for 25 cycles at 96^{o}C for 10sec, 50^{o}C for 5 sec and 60^{o}C for 4mins. The samples were sent to Warwick Life science Genomic Resource centre for sequencing. Results were viewed with the aid of Chromas V2.11 (Technelysium Pty Ltd) and the sequences were analysed in detail with the aid of DNASTAR Lasergene 11 software suit.

2.2.19 Linearization and extraction of recombinant plant virus vectors

Recombinant PVX vectors were first linearized by digestion with *Spec*I (New England Biolabs). The reaction was carried out in 100µl containing 30 Units of *Spec*I, 10µl of 10 x NEBuffer 4, 10µl of 10 x BSA (10 mg/ml), 10µg of miniprep DNA. The reaction was carried out at 37^{o}C for 3hours. An equal volume of phenol:chloroform:isoamyl alcohol 25:24:1 (100µl) was added to the digestion mixture and gently vortexed for 20-30seconds. The mixture was then centrifuged for 3 minutes at 15000 rpm and 100µl of upper phase was transferred to a new 1.5ml Eppendorf tube. An equal volume of chloroform:isoamyl alcohol 24:1 (Sigma) (100µl) was added. The tube was gently vortexed for 20-30seconds and centrifuged for 3minutes at 15000rpm. The supernatant was immediately transferred to a fresh 1.5ml tube. 10µl of 3M sodium acetate and 250µl of 100% ethanol was added to the tube. The resulting mixture was temporarily stored at -20^{o}C for an hour and then centrifuged at 15000 rpm at 4^{o}C for 18 minutes. Following washing in 70% ethanol, the pellet was air-dried and dissolved in 40µl of water. The final concentration of linearised DNA vector was 250ng/µl.

2.2.20 *In vitro* transcription for synthesis of infectious recombinant viral RNAs

In vitro transcription reaction was set up in a total volume of 100µl as follows; 20 µl nuclease-free water (Promega) 5µl of 10 x RNA Pol reaction buffer (400mM Tris –HCl, 60mM $MgCl_2$ at 25^{o}C, 1µl of 40 units/µl RNasin ribonuclease inhibitor (Promega), 5µl of 5mM m^7G (5')G RNA Cap structure Analog (New England Biolabs), 5µl of 10 x NTP4

(20mM each of ATP, CTP, UTP and 2mM GTP ; Roche), 10µl of 250 ng/µl linearised DNA template. 4µl of 50units of T7 polymerase (New England Biolabs) was added to the reaction mixture after incubation at 37^{o}C for 10 minutes. The reaction remained at 37^{o}C for a further 25 minutes, followed by the addition of 5µl of 20mM GTP at 37^{o}C for 35 minutes. The resulting mixture was treated with 1µl of RNase–Free DNase (Promega) to remove any template DNA. Purification of recombinant viral RNA transcripts was carried out as described in 2.2.10. RNA transcripts were dissolved in 20µl of nuclease-free water and used for subsequent inoculation of test plants.

2.2.21 Plant inoculation and maintenance

Plants were inoculated at the 5-6 leaf stage. For inoculation, two leaves from each plant was dusted with carborundum powder (Fisher Scientific) and then carefully rubbed with 10µl of *in vitro* synthesised RNA transcripts. After inoculation, the test plants together with Mock (water inoculated) control plants were watered and maintained in an insect-free containment glasshouse at 25^{o}C in constant LD (16hrs photoperiod). Double replicate experiment was carried out and data obtained from the latter was analysed and presented in this project.

2.2.22 RNA extraction from plant leaves

Total RNA was extracted from leaf material. The leaf tissue samples were frozen with liquid nitrogen and homogenised using a pestle and mortar. Samples were transferred into a 1.5ml Eppendorf tube with 1ml of TRIzol® Reagent (Invitrogen). For RNA purification the manufacturer's protocol was followed. RNA concentration and quality was measured with a NanoDrop™ ND-100 Spectrophotometer (Thermo Scientific) as described in 2.2.17. Total RNA was treated with TURBO DNA-free ™ DNase (Ambion) to remove any genomic DNA contaminants. Phenol-chloroform extraction was then carried out (see section 2.2.10). Pellet obtained was dissolved in 20 µl of nuclease –free water and used for subsequent RT-PCR analysis.

2.2.23 Software Tools

Data was statically analysed using GenStat 15th Edition 32bits. Nucleotide sequences were viewed with the aid of MegAlign (DNASTAR Lasgergene 11, Madison,WI) and MEGA5.1 while sequencing data was analysed using Chromas 2.23 (Technelysium Queensland, Australia). Oligos were designed with the aid of Primer3plus and synthesised by Fisher Scientific.

Chapter 3

The Expression of *Flowering Locus T* and It's orthologues in plants

Chapter 3: Expression of *FT* and *FT* orthologues in plants

3.1 Introduction

The transition between vegetative and reproductive growth phase is one of the crucial stages that plants undergo during post embryonic development. This biological process is regulated by a complex network pathway which is synchronised by both exogenous and endogenous factors (as described in chapter 1). In the model plant *Arabidopsis*, one of the widely studied genes involved in this process is *FT*. Interestingly the majority of the transcription factors regulating flowering have been documented to have diverged at some point but the *FT* gene is known to be evolutionary conserved amongst plant species (Wigge *et al*., 2011). *FT* research has undoubtedly been focused on its role in influencing the floral transition in plant but in recent years there has been clear evidence to suggest that *FT* signalling plays a wider role in plant's growth and development.

In *Arabidopsis*, *FT* regulates stomatal opening (Kinoshita *et al*., 2011), and meristem maintenance in cooperation with *SHOOT MERISTEMLESS* (*STM*) during inflorescence development (Smith *et al*., 2011). *FT* has been reported to also have a role in seed germination (Chiang *et al*., 2009). In tomato, *FT* homolog *SFT* has a role in stem growth, leaf maturation and architecture (Shalit *et al*., 2009). In poplar, the onset of bud dormancy is triggered by shorter days and lower temperatures, and this is dependent on a concomitant down-regulation in *FT* expression. Interestingly *FT*- overexpressing poplars do not set buds thus indicating that the CO-FT regulatory mechanism plays a crucial role in the process. In autumn, the buds set in and this involves the regulation of *PtFT1* (Bohlenius *et al.*, 2006).

In rice, *HD1* regulates spikelet number per panicle in concert through the up-regulation of two rice florigen genes *Rice Flowering-locus T1* and *Hd3a* (Endo-Higashi *et al*., 2011). In addition, increased tillering and accelerated flowering has been reported to occur in transgenic rice plants expressing green fluorescent protein–fused Hd31 from phloem-specific promoters (Tamaki *et al*., 2007). In onion flowering is induced by *AcFT2* while bulb formation is regulated by *AcFT1* (Lee *et al*., 2013). In potato the overexpression of rice *Hd3a* induced flowering and tuberization in non-inductive LD conditions (Navarro *et al*., 2011). It was also reported that potato *FT* orthologues *StSP6A* and *StSP3D* act as a tuberigen and florigen respectively.

Extensive research has been carried out on *FT* and its orthologues in order to develop new breeding strategies. Some plant species possess a long period of juvenile phase before becoming competent to flower (Mimida *et al*., 2009; Baurle *et al*., 2006; Huijser *et al*., 2011), for example it takes 6-10 years for poplar to initiate flowering (Flachowsky *et al*., 2009). It has been shown that *FT* and its orthologues could be used to shorten breeding cycles. In 2010, Zang *et al* reported that the expression of *Arabidopsis FT* driven by a heat-inducible promoter triggered early flowering in *Populus trichocarpa*. In addition stably transformed tobacco plants that expressed *FT* exhibited early flowering and seeds from transgenic plants flowered 39 days post germination while non-transgenic plants seeds flowered at 87-138 days post germination (Lewis *et al*., 2009). In apple, which has a juvenile phase of 5-12 years, it was demonstrated that the period of juvenility was significantly shortened. Transient expression of *Arabidopsis FT* using *Apple latent spherical virus* (*ALSV*) caused inoculated apple plants to flower 1.5 months post inoculation (Yamagishi *et al*., 2011; Flachowsky *et al*., 2012).

These results indicate that *FT* does have a wider role than initially envisaged. Thus investigating the effect of expression of the gene in diverse plant species could shed more light on its role in plant's growth and development.

In this chapter, I aim to investigate:

- Expression of *Arabidopsis FT* in Tobacco and its effect on flowering time.
- Expression of *Arabidopsis FT* and Tomato *FT* genes in Potato and its effect on tuberisation.
- Expression of *Arabidopsis FT* and Tomato *FT* genes in Tomato.
- Expression of *Arabidopsis FT* and Tomato *FT* genes in Brassica.

3.2 Materials and Methods

3.2.1 *In vitro* transcription and inoculation of *N. benthamiana*

After linearization of the recombinant PVX plasmids harbouring the cloned inserts, *in vitro* transcription was carried out (see 2.2.20). RNA transcripts were used to inoculate *N. benthamiana* plants at the 5-6 leaf stage. Leaf tissues that exhibited symptoms of systemic viral infection such as chlorosis (Fig 3.1) were harvested, freeze-dried and stored at -80^{o}C. The leaf tissue material was used as an inoculum source for subsequent experiments.

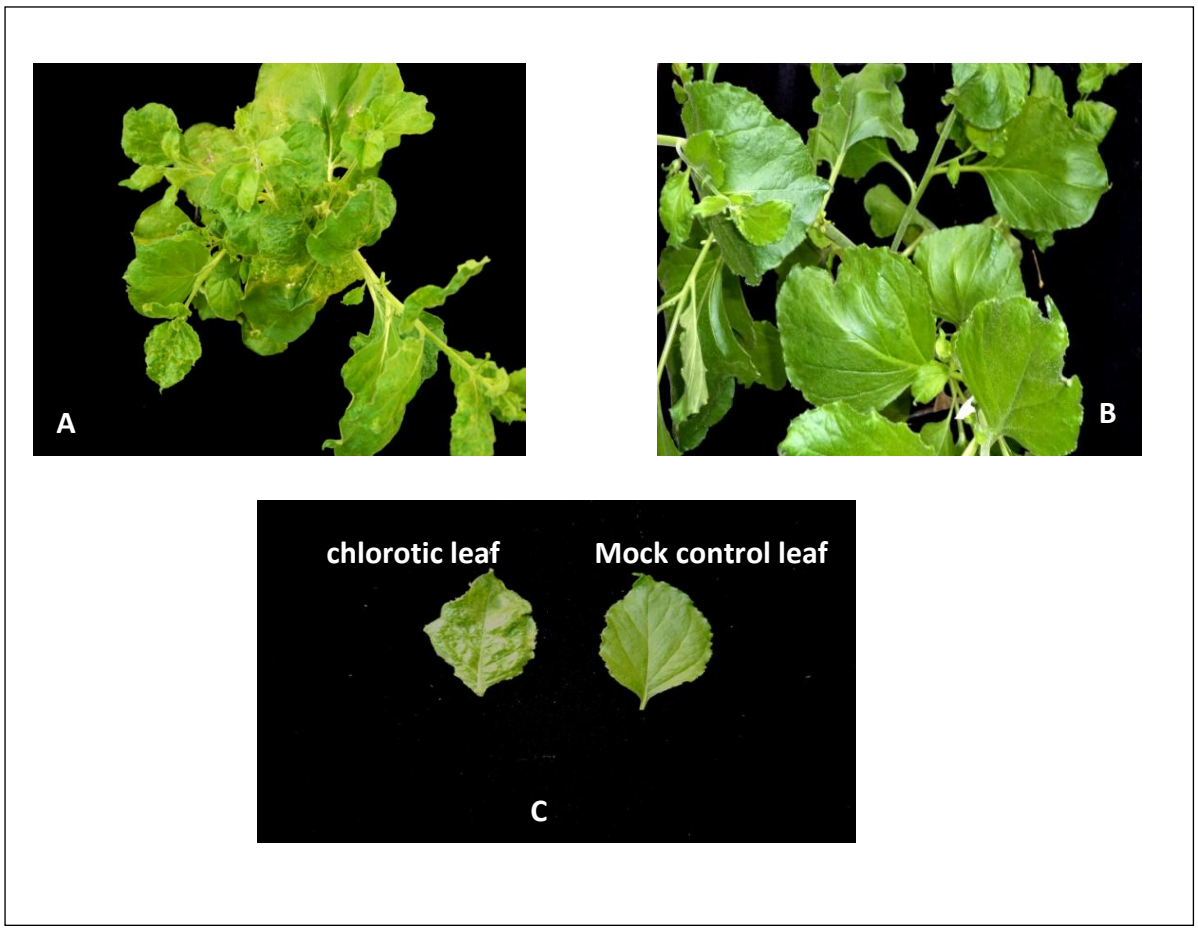


Figure 3.1. A. shows a PVX/FT infected *N. benthamiana* plant exhibiting viral infection symptoms. **B.** shows a mock –infected *N. benthamiana* plant and **C.** shows a virally infected *N. benthamiana* leaf and a mock -infected *N. benthamiana* leaf.

3.2.2 Virus –based flowering assay

High titre virus inoculum used in experiments was obtained by grinding approximately 200mg of viral infected leaf tissue in 600-1000µl of EB buffer (Qiagen). The resulting leaf sap containing virus particles was used to inoculate the plants. Phenotypic changes such as flowering time, stem length, seed number, lateral side shoot length and fruit weight was recorded. 5-10 young plants were mock inoculated with water or treated with PVX/FT, PVX/mFT, PVX/SP2I, PVX/SP5G, PVX/SP6A, PVX/FT-FLAG and maintained in LD conditions. Statistical analysis such as ANOVA was carried out on data obtained.

3.3 Experimental Results

3.3.1 Construction of PVX expression vectors

The tomato *FT* orthologue genes; *SP2I*, *SP5G* and *SP6A* and His and FLAG tagged wild-type *Arabidopsis FT* were PCR amplified using high fidelity KOD polymerase and the primers SP2F/SP2R, SP5F/SP5R, SP6F/SP6R, FT-HIS F/ FT-HIS R, HIS-FT F/HIS-FT R, FT-FLAG F/FT-FLAG R (see appendix for all primer list). Amplified PCR products (Fig 3.2) were sequenced and cloned into the *EagI* and *SalI* sites of PVX (Fig 3.3). The recombinant plasmids PVX/mFT, PVX/FTC4, PVX/FT and PVX/GFP used in this chapter were kindly provided by Prof Yiguo Hong (See Fig 1.9; chapter 1). The mutant *FT (*m*FT)* construct contained a non-translatable version of *FT* in which the start codon (ATG) had been replaced with a stop codon (TAG). The *FT* C4 construct was derived from the native *Arabidopsis FT*. *FT*C4 contained a point mutation within the Phosphatidylethanolamine-Binding protein (PEBP) domain of *Arabidopsis FT*. The mutation caused an amino acid change from Valine to Alanine. This construct in particular has been documented to induce increased seed production in tobacco (*N.tabacum*) as well as triggering early flowering (Li *et al*., 2009). This construct was used in order to determine the effect of this particular construct on seed production and flowering time of Ailsa Craig tomato plants. The tomato *FT* clones *SP2I*, *SP5G* and *SP6A* were used in order to investigate their effects on phenotypic characterization e.g *SP2I* has been reported to have possible roles in the development of vegetative and reproductive organs.

Figure 3.2. PCR amplification of DNA. **Gel A** represents DNA products obtained from amplification for FT-His, His-FT and FT-FLAG. Lane 1 contains 5ul of 1kb ladder marker (Invitrogen), lanes 2-4 shows positive PCR products for FT-His, His-FT and FT-FLAG DNA using FT-HIS F/ FT-HIS R, HIS-FT F/HIS-FT R, FT-FLAG F/FT-FLAG R. **Gel B** represents DNA products obtained from amplification for *SP2I*, *SP5G* and *SP6A*. Lane 1 contains 5ul of 1kb ladder marker (Invitrogen), lanes 2-4 shows positive PCR products for *SP5G*, *SP2I* and *SP6A* DNA using SP5GF, SP5GR, SP2IF,SP2I, SP6F and SP6R gene specific primers

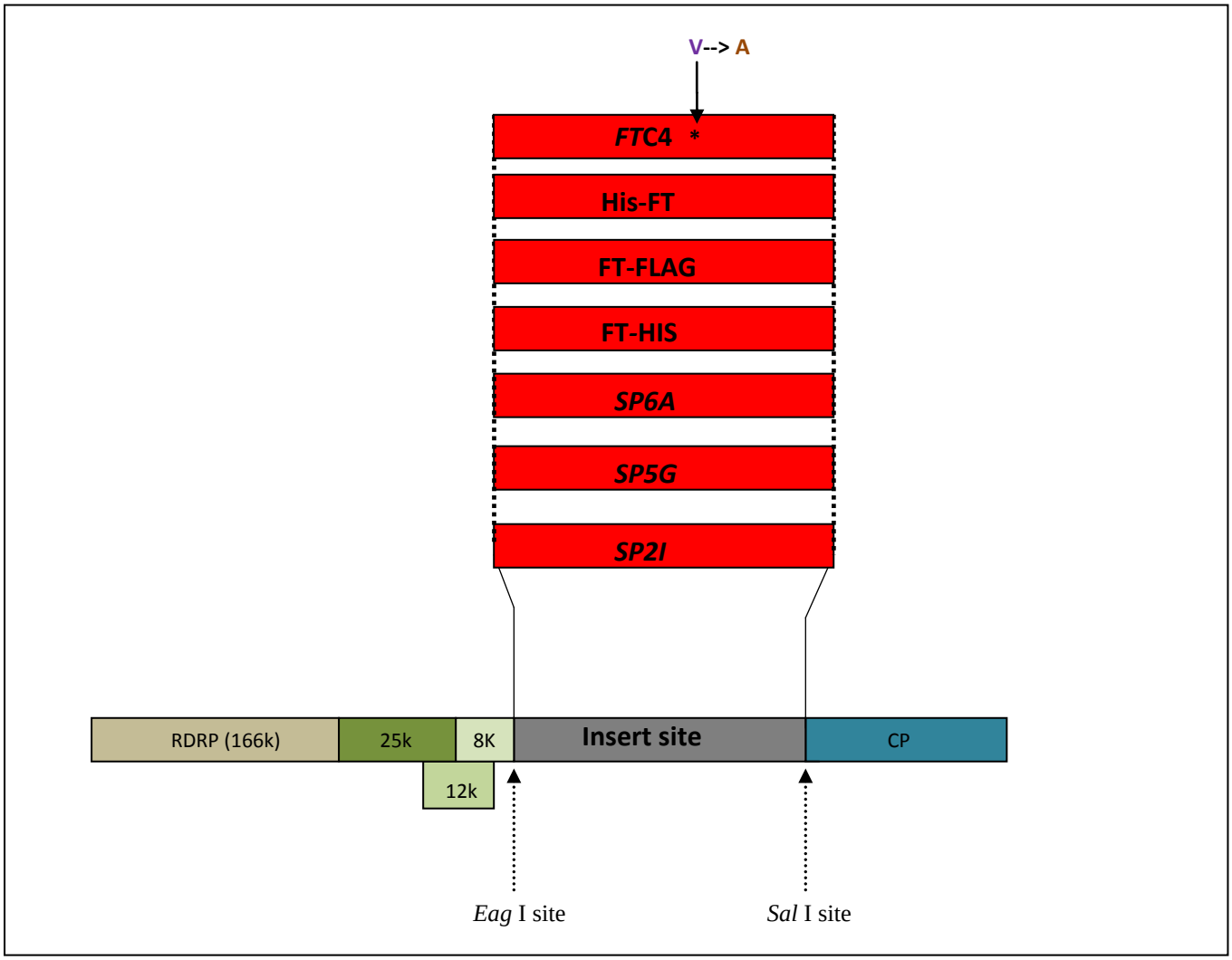


Figure 3.3 A schematic representation of the expression constructs used in the experiment. Recombinant plasmids were linearised with *SpeI* prior to *in vitro* transcription. The RNA dependent RNA polymerase (RDRP) (166K) is involved in promoting PVX replication while the Coat protein (CP) is involved in cell to cell movement and systemic spread in host plant. The tomato *FT* orthologues; *SELF PRUNING 2I (SP2I), SELF PRUNING 5G (SP5G), SELF PRUNING 6A (SP6A)*, FTC4 contained a point mutation within the Phosphatidylethanolamine-binding protein (PEBP) domain which conferred an amino acid change from Valine to Alanine. WT *Flowering locus T* (*FT*) tagged with polyhistidine (His) and FLAG octapeptide respectively were cloned into the *Eag I* and *SaI 1* restriction sites.

3.3.2 Expression of *Arabidopsis FT* in Maryland Mammoth tobacco under non-inductive LD condition

Young tobacco plants were maintained and inoculated at the 5-6 leaf stage. Inoculation of plants was carried out with sap inoculum. At 7 days post inoculation, visible viral infection symptoms were observed on the young leaves of all the PVX/FT, PVX/mFT and PVX/GFP inoculated plants but not mock-inoculated plants. This provided an indication that the host plants had been successfully infected. The symptoms included the appearance of yellow patches (chlorosis) on the leaves (Fig 3.4; Red arrows). The leaves also appeared shrivelled around the edges. All control plants appeared healthy as they were not infected with the virus or by other pathogens. At 23 days post inoculation it was observed that the PVX/FT inoculated plants had started to bolt (Figure 3.5). Bolting was characterized by a distinct increase in stem length. It was also observed that the stem length of the other plants inoculated with; PVX/mFT, PVX/GFP and mock inoculated plants remained short. This is because these plants remained in their vegetative stage, which is typically characterised by a relatively slow stem growth and an increase in leaf surface area, in non-inductive LD conditions.

At 30 days post inoculation all the PVX/FT inoculated plants had developed floral buds. The stem length at this stage was 36cm which was more than a 5 fold increase over the stem length of PVX/mFT, PVX/GFP and mock inoculated plants which were 2cm, 1cm and 1cm respectively.

At 44 days post inoculation it was observed that the floral buds of all the PVX/FT inoculated plants had opened. The flowers can be clearly seen in figure 3.6. The mean stem length at this stage was 60cm (Fig 3.7). Other test plant subjects continued to remain in their vegetative stage which was marked by further increase in their leaf surface area. The PVX/FT inoculated plants flowered early because the transition to the reproductive phase had been triggered by viral expression of the *Arabidopsis FT* even though they were being grown in non-inductive LD conditions.

Figure 3.4. Young SD *N. tabacum* Maryland Mammoth at 7 day post inoculation. Inoculated plants exhibiting viral infection symptoms. The red arrows indicate the chlorotic lesions spread across the surface of a systemic leaf.

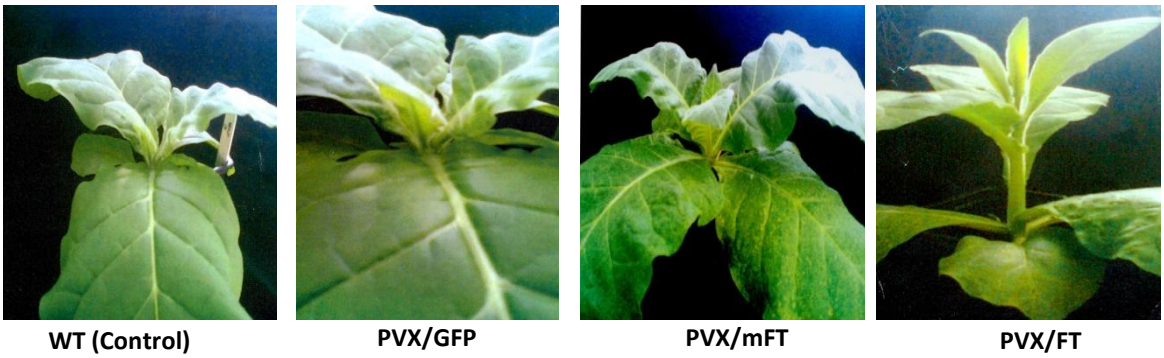


Figure 3.5. Young SD *N. tabacum* Maryland Mammoth plants at 23 days post inoculation.

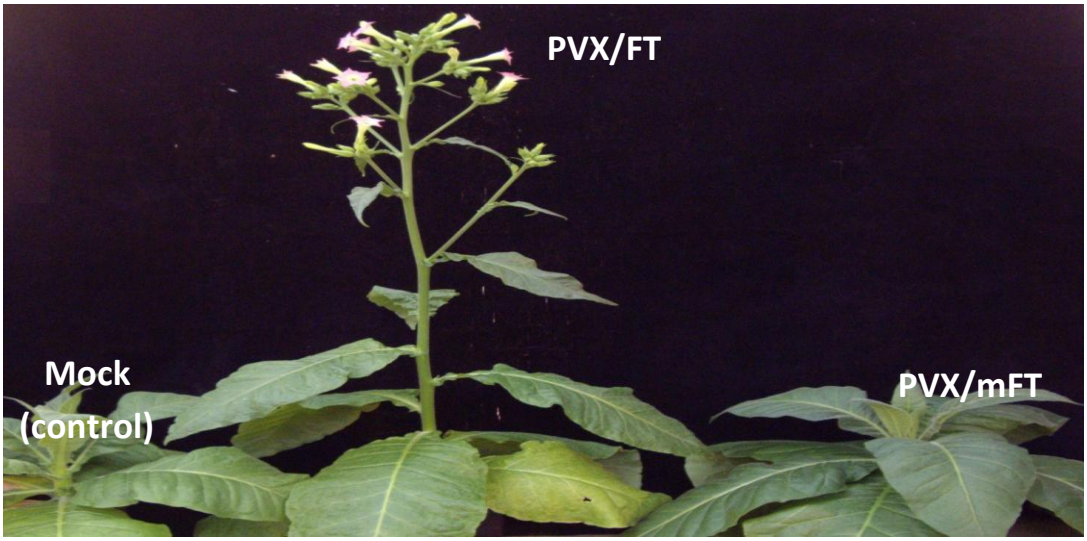


Figure 3.6 *N. tabacum* Maryland Mammoth plants at 44 days post inoculation. PVX/FT inoculated plant (middle) showing early flowering while mock (control) and PVX/mFT inoculated plants remained in vegetative stage.

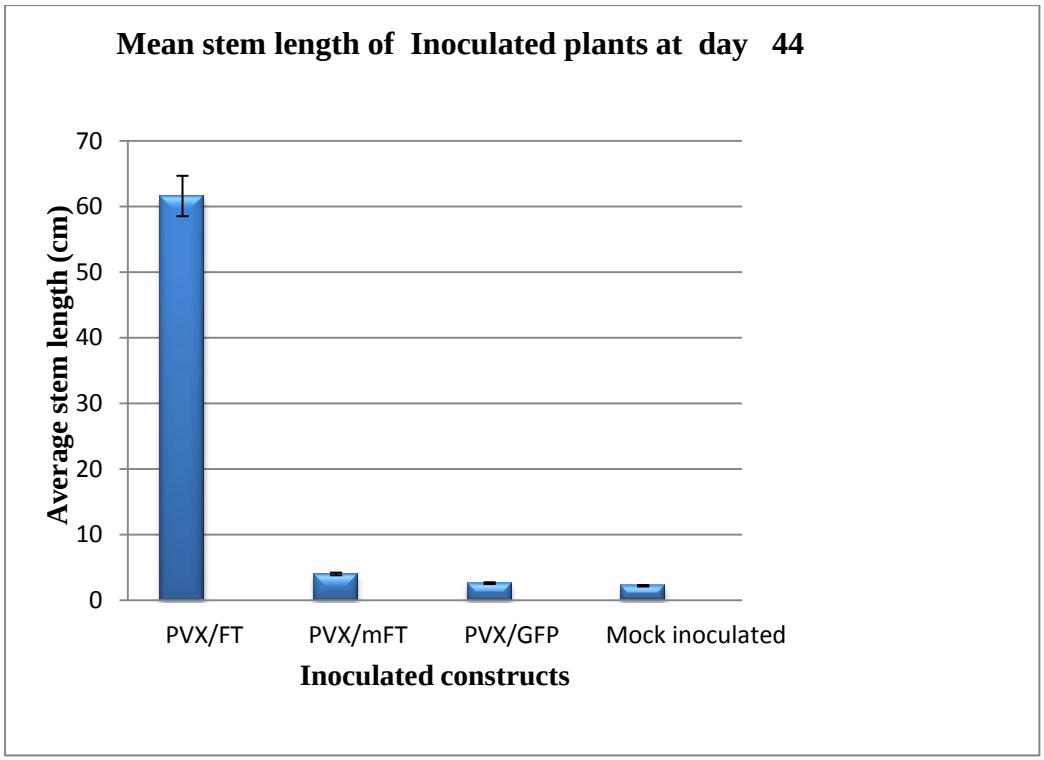


Figure 3.7 The average stem length (cm) for each test plant group 44 days post inoculation. Error bars indicates the Standard error. (n=5).

3.3.3 Expression of *Arabidopsis FT* in potato under non-inductive LD condition

At 30 days post inoculation, visible viral infection symptoms were observed on the young leaves of the PVX/FT inoculated potato plants. This provided an indication that the host plants had been successfully inoculated/infected. The symptoms however appeared mild despite PVX being a potato virus. The most prominent symptom was the development of necrotic-like lesions on the leaves (Fig 3.8). RT-PCR was carried out on systemic leaf samples to check if the virally expressed *Arabidopsis FT* was present in the leaves as the visible symptoms were mild. Test results confirmed that the virally expressed *Arabidopsis FT* was indeed present in the systemic leaves of the inoculated potato plants (Fig 3.9a).

At 40 days post inoculation one out of five of the PVX/FT inoculated plants had tuberized (Fig 3.10a) while none of the control plants tuberized (Fig 3.10b). Tissue samples were harvested from the tuber, stolon and young leaves to investigate if the *Arabidopsis FT* RNA was present in the samples. RT-PCR test confirmed that the *Arabidopsis FT* RNA was present in the tissue samples (Fig 3.9 b). The data obtained from this experiment would not

reflect an accurate representation of the effect of the target gene overexpression on tuberization because only one out of five of the potato plants tuberized therefore in order to obtain a more reliable percentage accuracy more plants could be used e.g. 20-30 plants per test group.

Figure 3.8 Young potato plants. Viral infection symptoms seen on the PVX/FT inoculated plant (Red arrows).

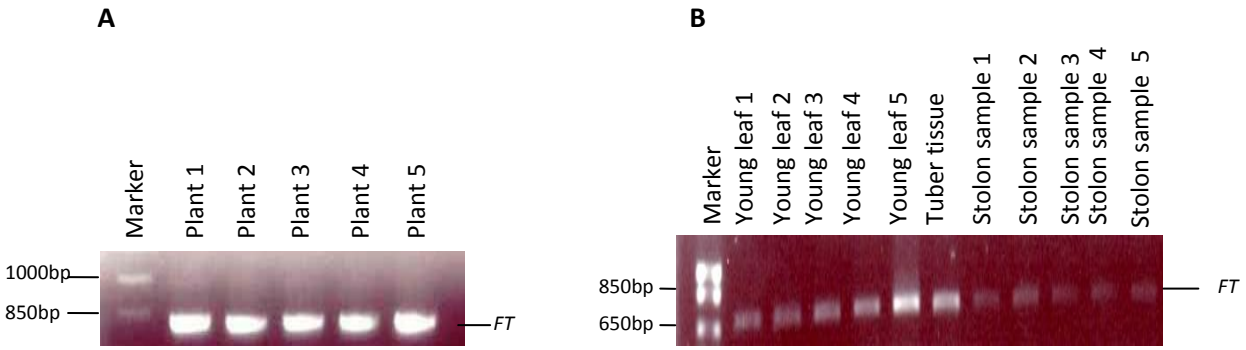
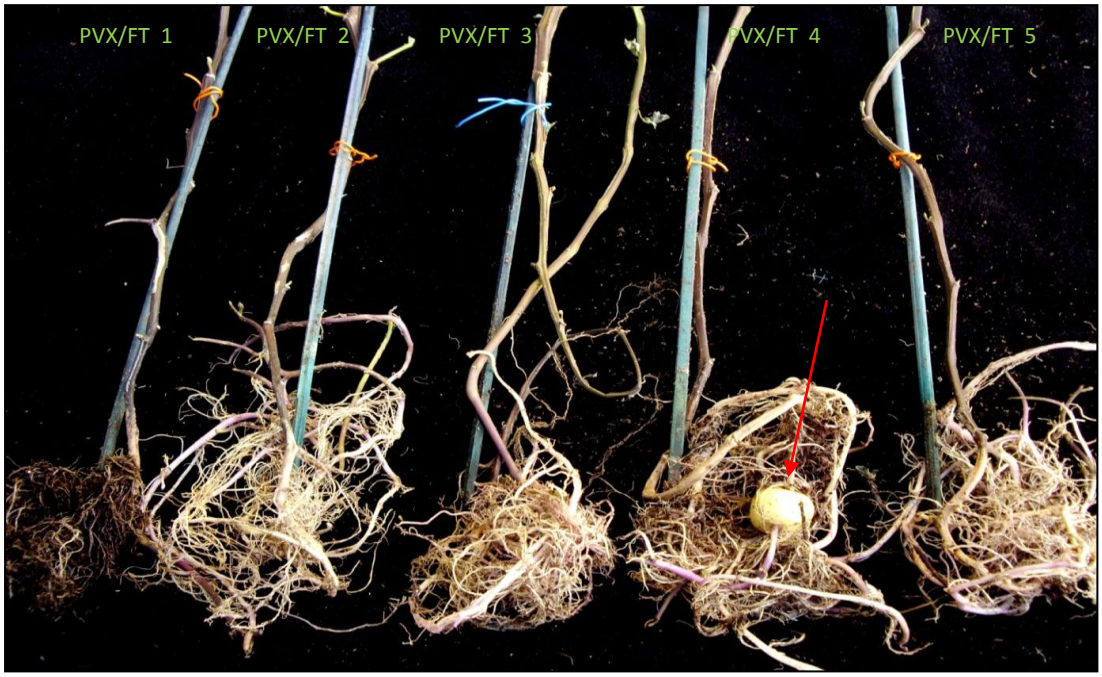


Figure 3.9. RT-PCR detection of *Arabidopsis FT* RNA in potato tissue samples.**Gel A** represents young systemic leaf samples obtained from PVX/FT inoculated potato plants at 30 days. Lane 1 contains 5ul of 1kb ladder marker (Invitrogen), lanes 2-6 shows positive PCR products for PVX/*Arabidopsis FT* DNA (750bp) detected in the young leaves harvested from test plants 1-5 respectively using primers PP82 (►) and PP356 (◄). **Gel B** represents tissue sample harvested from PVX/FT inoculated potato plants at the end of the experiment (90days). Lane 1 contains 5ul of 1kb plus ladder marker (Invitrogen), Lanes 2-6 in young leaves and Lanes 7-12 potato tuber tissue and stolon tissue respectively. PCR bands were confirmed by sequencing using gene specific primers.

A



B

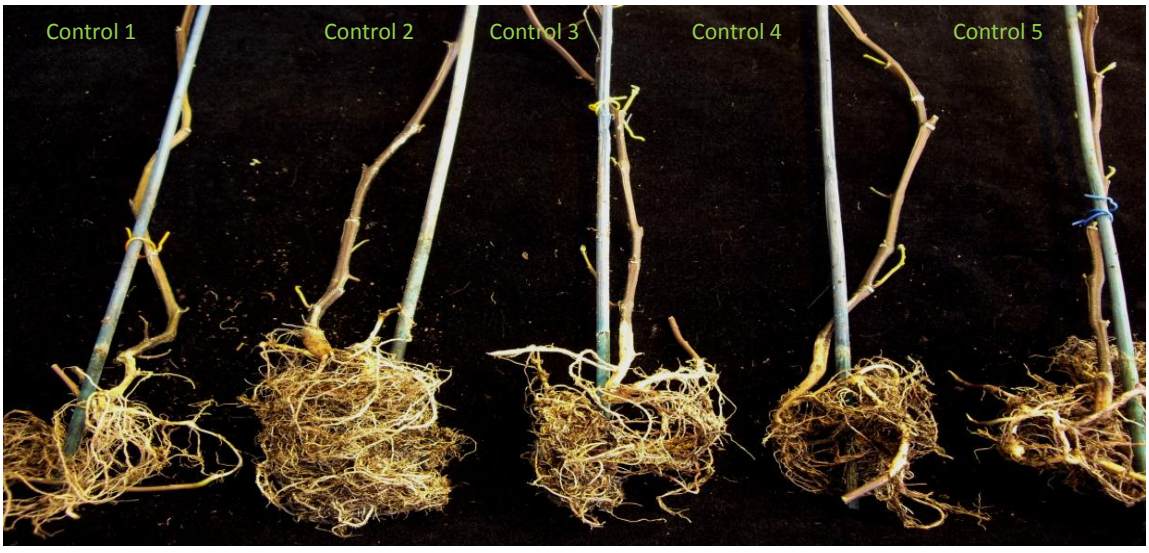


Figure 3.10. (**A)** Underground parts of *Andigena 7540* PVX/FT inoculated plants 1-5 at 40 days post inoculation . The red arrow shows a potato tuber. (**B**) Underground parts of *Andigena 7540* control plants 1-5 at 40 days post inoculation.

3.3.4 Expression of *Arabidopsis FT* and *FT* orthologues in tomato

At 14 days post inoculation visible viral symptoms were observed on the young leaves of all the PVX/FT, PVX/FTC4, PVX/mFT, PVX/GFP, PVX/SP2I, PVX/SP5G, PVX/SP6A and PVX/FT-FLAG inoculated test plants. The most prominent of which was the presence of yellow patches (chlorosis) (Fig 3.11). All control (mock-inoculated) plants remained healthy as they were not infected with the virus.

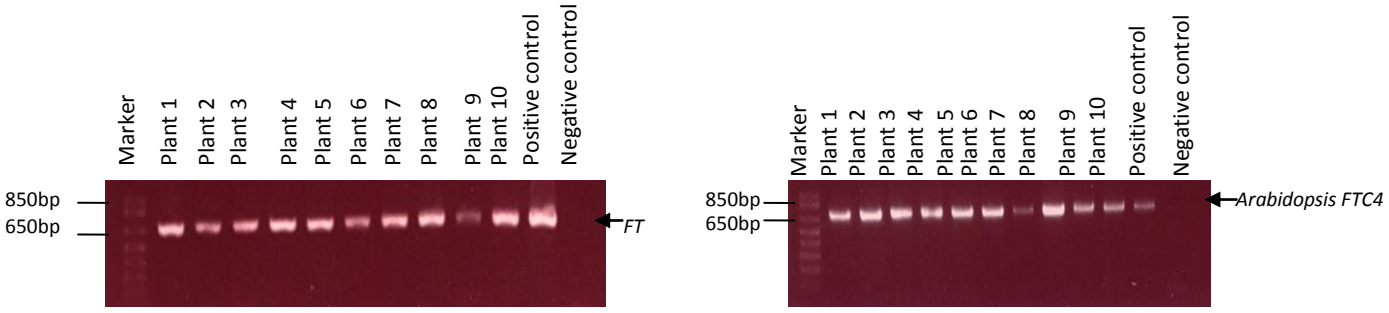
Young systemic leaves were harvested from all 10 tomato plants per test group that were inoculated with the PVX/FT, PVX/FTC4, PVX/mFT, PVX/SP2I, PVX/SP5G, PVX/SP6A and PVX/FT-FLAG. RT-PCR of these samples confirmed that the PVX RNA transcript was present in systemic leaf tissue from all inoculated plants (Fig 3.12) except for plants inoculated with the *SP2I* and *SP5G* constructs which was negative.

At 21 days post inoculation it was observed that the PVX/FT and the rest of the test plant group which included PVX/FTC4, PVX/mFT, PVX/SP2I, PVX/SP5G, PVX/SP6A and PVX/FT-FLAG inoculated tomato plants had developed floral buds. The PVX/GFP and Mock (control) plants however developed floral buds a day later at 22 days. At 34 days post inoculation the PVX/FT-FLAG, PVX/SP2I plants had open flowers while the PVX/FT, PVX/mFT, PVX/SP6A plants had open flowers a day later at 35 days and PVX/GFP and mock (control) plants flowered 3 days later at 37days (Fig 3.13A). Surprisingly the FTC4 inoculated plants flowered at 36 days, a day later than the PVX/FT plants which indicated that perhaps the mutation didn't have the same effect on promoting early flowering in tomato as it did in tobacco. It was observed that there was no significant difference in the number of days taken to develop buds and flower between the test plants and control plants as seen in Figure 3.13 (A).

There was a significant difference in the number of seeds produced between the PVX/FT, PVX/FTC4, PVX/SP2I, PVX/FT-FLAG and PVX/SP5G lines compared to control plants (Figure 3.13B). There was a significant difference in fruit weight between the PVX/FT, PVX/SP2I and PVX/FT-FLAG compared to the control plants (Fig 3.13C). It was also observed that there was a significant difference in seed weight between the PVX/FT, PVX/mFT and PVX/GFP inoculated plants compared to the controls (Fig 3.13D). The mean seed weight was 0.055g, 0.056g and 0.054g respectively compared to the Mock control plant's seed weight which was 0.042g. This was quite surprisingly because the mutant *FT* and GFP constructs were used as controls. A plausible explanation for this occurrence could be

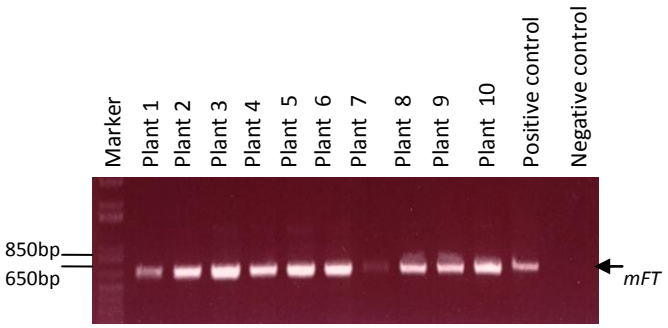
due to components within the PVX virus having an interaction in seed weight development in the infected plant.

Figure 3.11 Young Ailsa Craig tomato plants at 14 days post inoculation. Plants exhibiting viral infection symptoms. The red arrow indicates the chlorotic lesion spread across the surface of a systemic leaf.

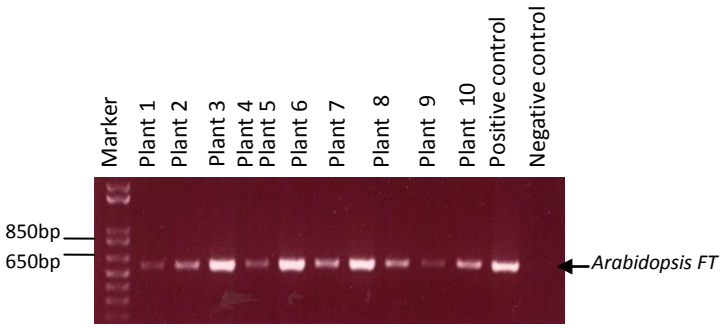


A. FT gel

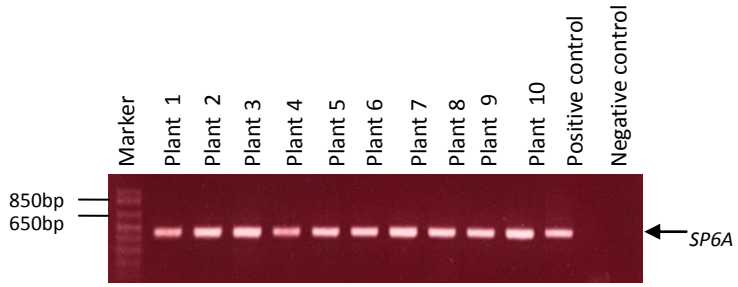
B. FT C4 gel



C. mFT gel



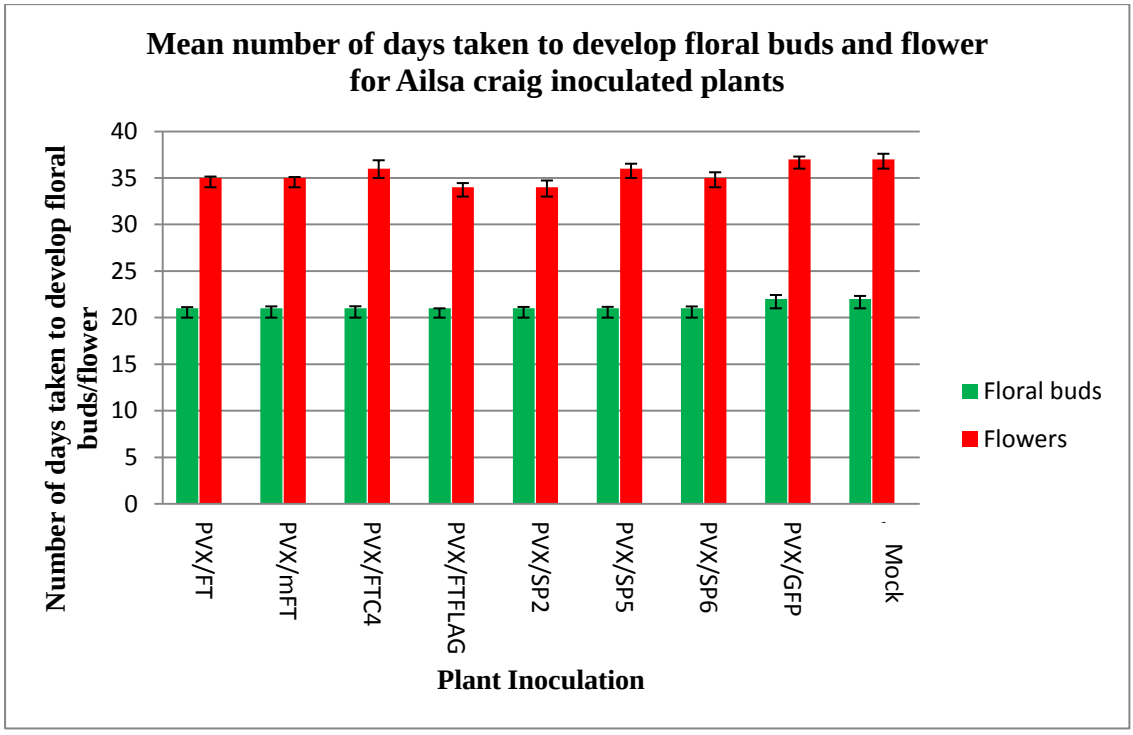
D. FT- FLAG gel



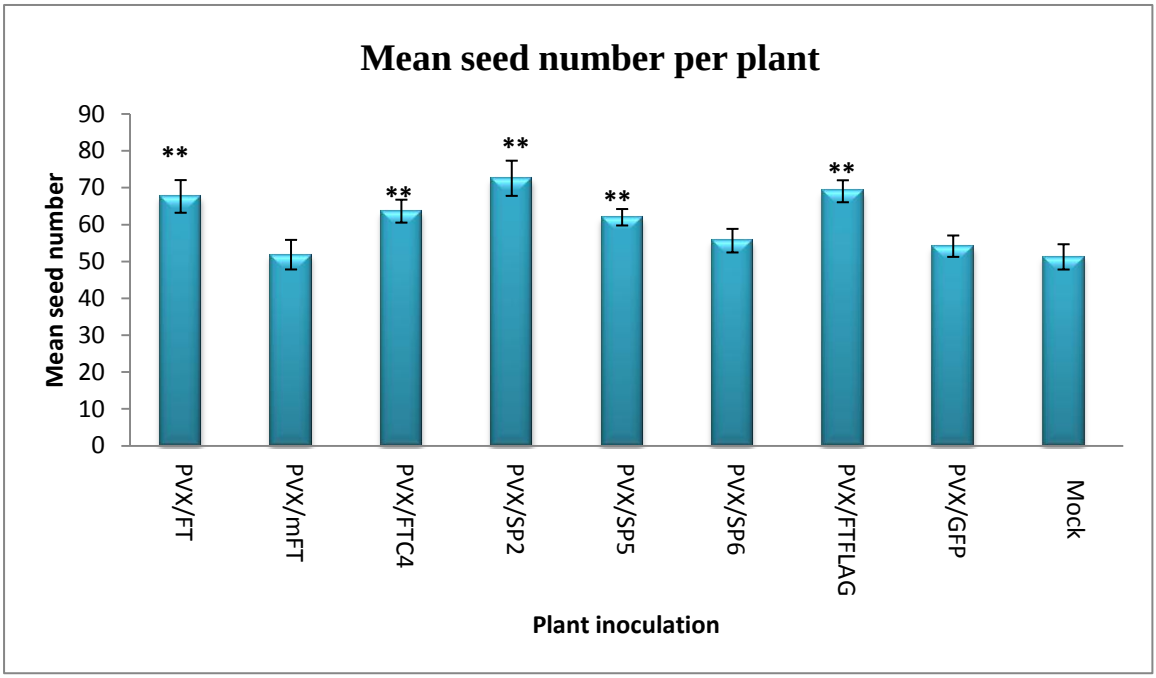
E. SP6G gel

Figure 3.12 RT-PCR detection of *Arabidopsis FT*, m*FT* , FT C4 and FT-FLAG and *SP6A* RNA in systemic leaf tissue samples of inoculated Ailsa Craig tomato plants. 5µl of 1kb plus ladder marker (invitrogen) was used. PVX specific forward primer PP82 (►) and the respective gene specific reverse primer was used. Positive control used in each lane was the respective recombinant plasmids while water (as template) was used in negative control.

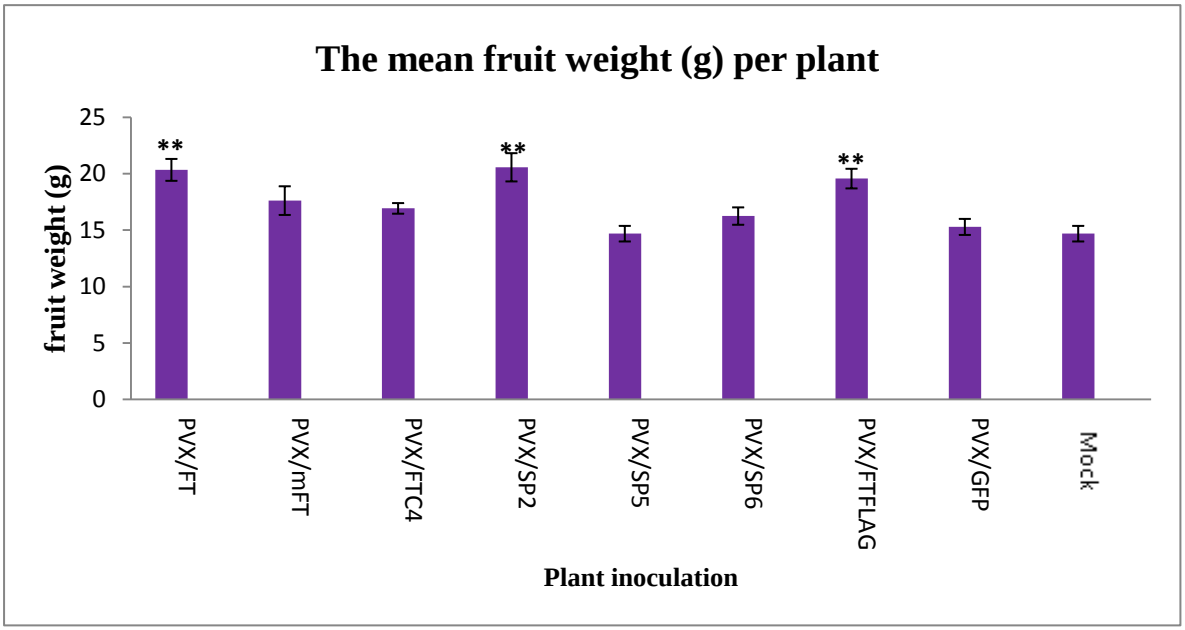
A


Mean number of days taken to develop floral buds and flower for Ailsa craig inoculated plants
Number of days taken to develop floral buds/flower
40
35
30
25
20
15
10
5
0
PVX/FT
PVX/mFT
PVX/FTC4
PVX/FTFLAG
PVX/SP2
PVX/SP5
PVX/SP6
PVX/GFP
Mock
Floral buds
Flowers
Plant Inoculation

B


Mean seed number per plant
Mean seed number
90
80
70
60
50
40
30
20
10
0
**
PVX/FT
PVX/mFT
PVX/FTC4
PVX/SP2
PVX/SP5
PVX/SP6
PVX/FTFLAG
PVX/GFP
Mock
Plant inoculation

C



D

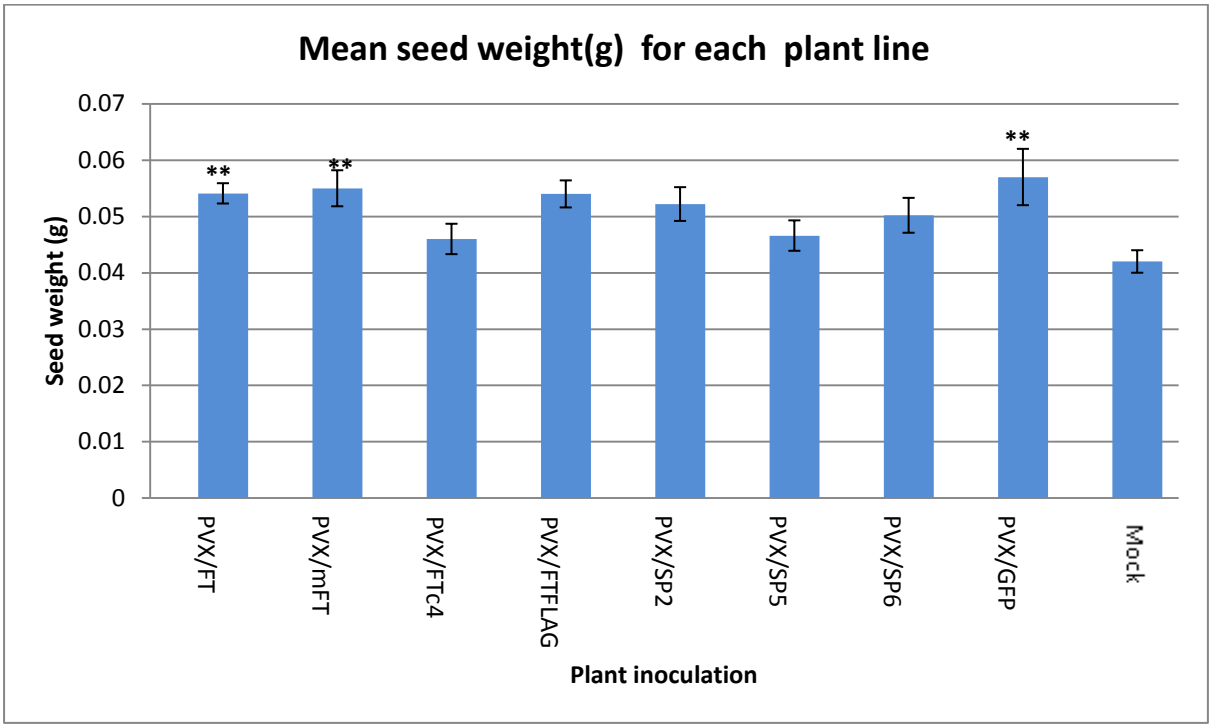


Figure 3.13 Effect of expression of *FT* and *FT* orthologues on tomato. (A) Shows the mean number of days taken to develop floral buds and flowers for Ailsa craig inoculated plants (B) Shows the mean seed number produced per plant. (C) Shows the mean fruit weight (g) per plant for Ailsa craig inoculated plants (D) Shows the mean seed weight (g) per plant. (n=10). Error bars indicate the standard error. P values; * = $P < 0.05$, ** = $P < 0.01$, *** = $P<0.001$).

3.3.3.1 Effect of *Arabidopsis FT* and *FT* orthologues on lateral side shoot development in tomato

10 tomato plants per construct were used in this experiment. The reference lateral shoot length used throughout the duration of the experiment was 4cm. This was determined after carrying out a pilot experiment. All plants were inoculated at the 5-6 leaf stage and maintained under the same LD condition. At 60 days post inoculation lateral side shoot development was observed on the PVX/FT, PVX/FTC4, PVX/SP2I and PVX/SP5G inoculated tomato plants compared to the PVX/mFT, PVX/GFP and mock (control) plants (Fig 3.14; red column bars).

LSS development was observed at 87 days post inoculation as time progressed (Fig 3.14 yellow column bars). At 87dpi the PVX/SP2 inoculated plants had the highest total number of LSS of 20 while the PVX/FT inoculated plants had a total LSS number of 19. The PVX/FT-FLAG, PVX/FTC4 and PVX/SP5G inoculated plants had a total LSS of 13, 16, and 17 while the PVX/mFT, PVX/SP6A, control (mock inoculated) and PVX/GFP plants had lower numbers of LSS of 8, 6, 4 and 4 respectively (Fig 3.14; yellow column bars).

At 101dpi increased LSS development was observed. There was a significant difference in the total number of LSS between the PVX/FT, PVX/FTC4, FT-FLAG, PVX/SP2 and PVX/SP5 compared to the controls (Fig 3.14; blue column bars). The total LSS number was 24, 23, 24, 23, 29 and 26 respectively compared to the controls; PVX/mFT, PVX/GFP and Mock inoculated which were 15, 9 and 11 respectively. This was an interesting discovery as there is to date no report of the effect of *Arabidopsi*s *FT* or *FT* orthologue on lateral side shoot development in tomato. Unpublished data from a research group consortium based in Spain (CNB) reported development of extended lateral side shoots in potato plants that were expressing the rice *FT* (*Hd3a*). In 2012, Hiraoka *et al* reported that *FT* and *TSF* were involved in modulating lateral shoot development in *Arabidopsis* (Hiraoka *et al*., 2012).

These observations suggested that the *FT* gene may play a role in lateral side development in some plant species. LSS development in tomato has long been believed to be regulated by hormonal balance in the plant, with auxin and abscisic acid (ABA) both playing pivotal roles (Tucker 1976). The mechanism of action is said to be indirect. Auxin induces the formation of abscisic acid in the stem tissue. The accumulated ABA then migrates into the bud tissues and inhibits bud growth (Tucker 1977). Elaborate experiments would need to be carried out to understand the role that *FT* plays in initiating lateral shoot development.

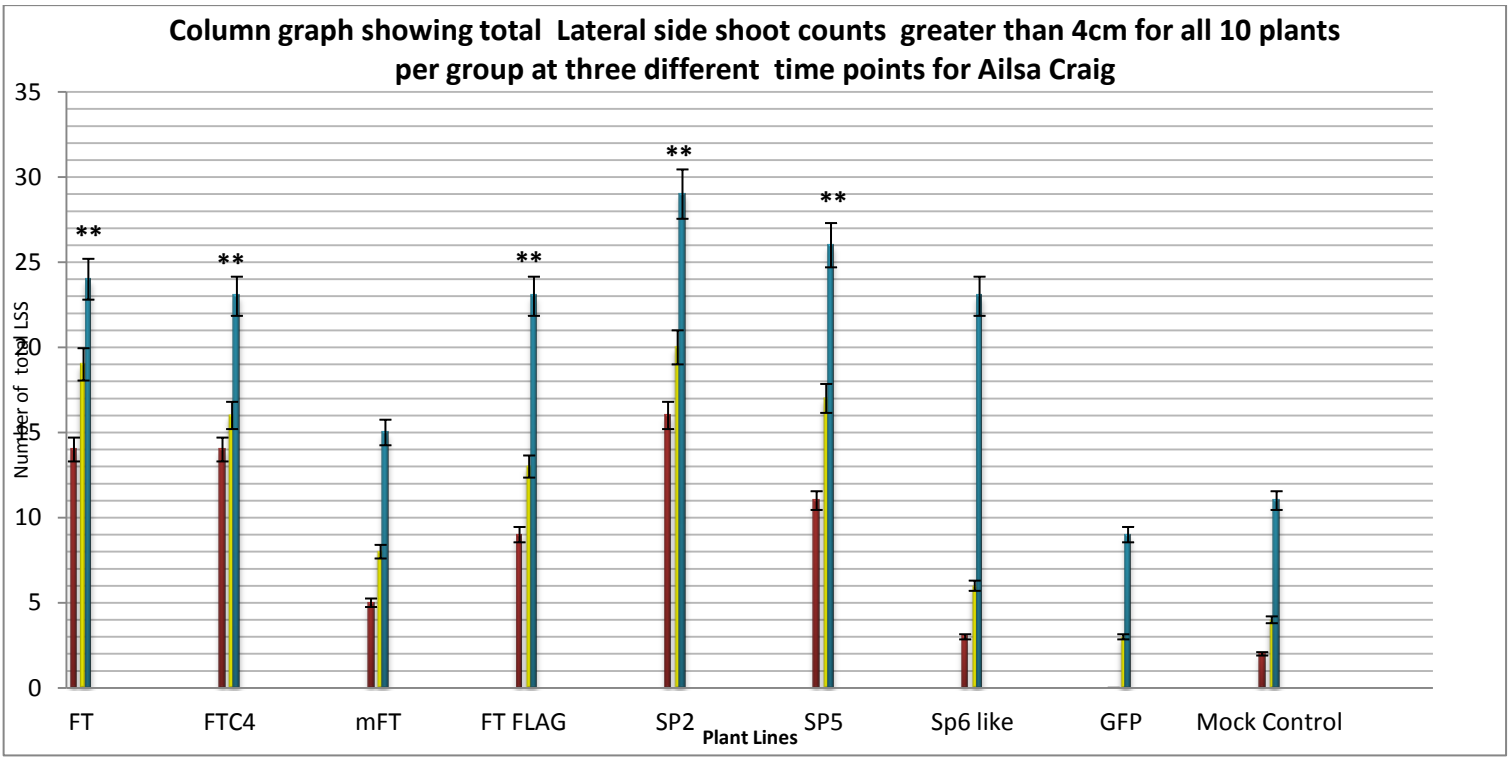


Figure 3.14 Effect of *FT* and *FT* orthologues on tomato lateral side shoot development. The graph above shows the total number of lateral side shoots per plant 10 plants per test group at 60dpi (red column bar), 87dpi(yellow column bar) and 101dpi (blue column bar). Error bars indicate standard error. P values; * = $P < 0.05$, ** = $P < 0.01$, *** = $P<0.001$).

3.3.5 Expression of *Arabidopsis FT* in Brassica

Five brassica plants per construct were used in this experiment. All plants were inoculated at the 5-6 leaf stage and maintained under the same LD condition. At 18 days post inoculation, visible viral infection symptoms were observed on the young systemic leaves of both the PVX/FT and PVX/mFT inoculated plants. The symptoms were however very mild. The most obvious symptom was the presence of white patches sparsely spread across the leaves.

RT- PCR was carried out on young leaf samples to confirm if the virally expressed *Arabidopsis FT* RNA was present systemically throughout the plant. Results indicated that the *Arabidopsis FT* RNA was indeed present in the systemic leaves of the inoculated brassica plants. All mock (control) plants appeared healthy as they were not infected with the virus (Fig 3.15).

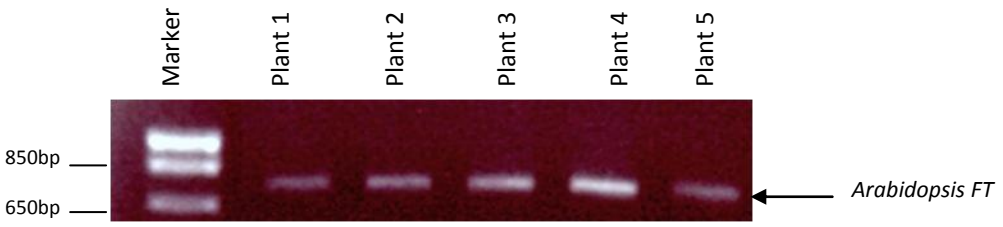


Figure 3.15. *Arabidopsis FT* RNA in *Brassica oleracea* var.*italica* young leaf tissue samples. Lane 1 contains 5ul of 1kb plus ladder marker (Invitrogen), Lane 2-6 contains positive PCR products for *Arabidopsis FT* RNA detected in young leaf samples obtained from inoculated brassica plants 1-5 using primers PVX primer PP82 (►) and *FT* specific primer PP356 (◄)

At 94 days post inoculation all of the control mock inoculated plants had flowered, two from five of the PVX/mFT had flowered. None of the PVX/FT inoculated plants had flowered (Fig 3.16). This was quite surprising as one would have expected the PVX/FT inoculated plants to flower earlier than the other test group subjects. A plausible explanation for this is because the Brassica and *Arabidopsis FT* sequences are so similar that the virally

expressed *Arabidopsis FT* RNA may have induced virus induced gene silencing (VIGS) of the endogenous Brassica *FT* gene expression.

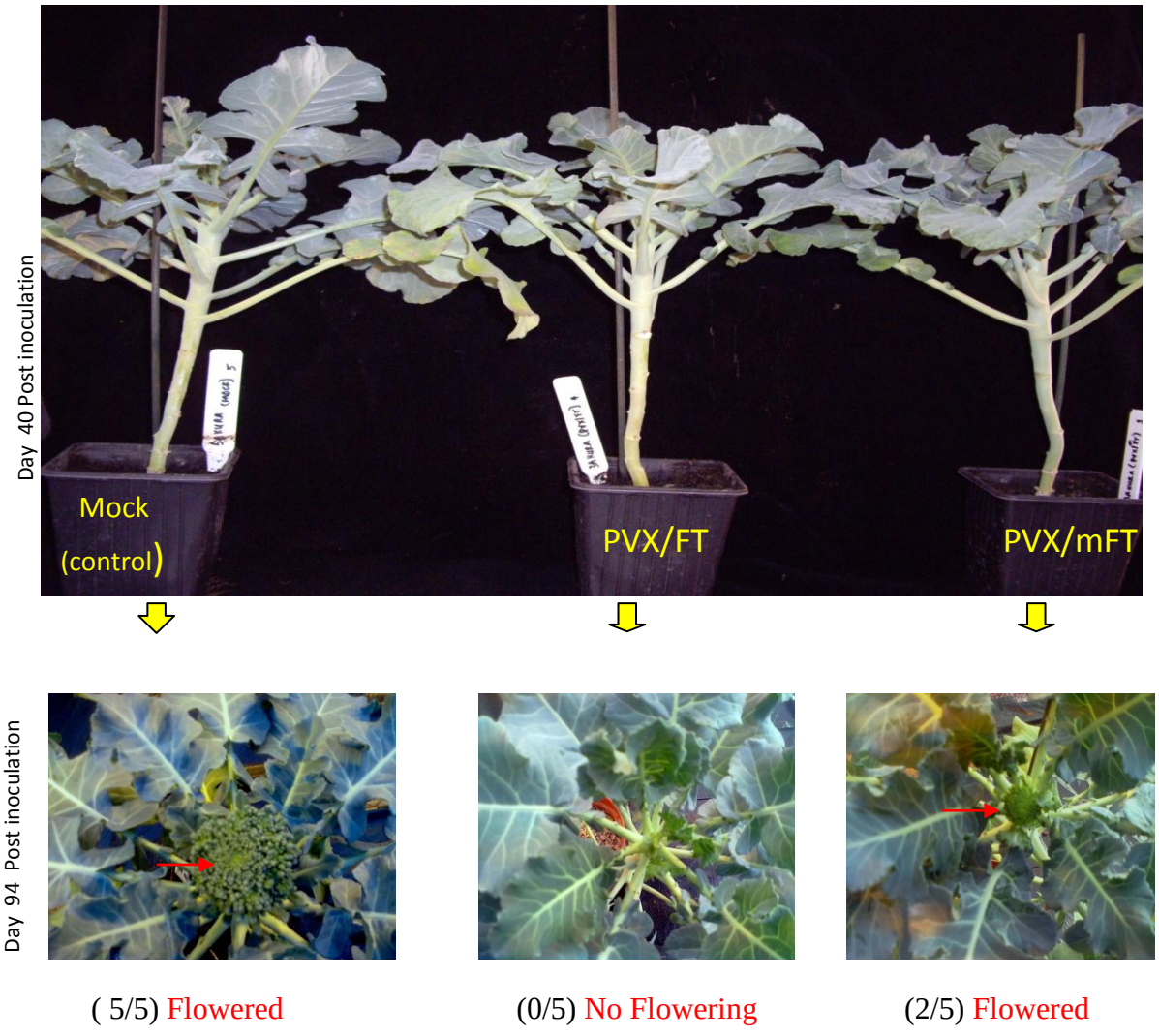


Figure 3.16. *Brassica oleracea* var.*italica* Marathon at 94 days post inoculation. The red arrows show the flower head .

A follow up experiment was carried out in order to investigate whether overexpression of the tomato *FT* gene *SP6A* in Brassica would induce early flowering. Phylogenetic analysis (Fig 3.17) shows that the tomato *FT* gene sequence is not as closely related to the Brassica *FT* sequence as the *Arabidopsis FT* gene therefore it was of interest to determine the effect of this gene being expressed in Brassica as it was less likely to induce VIGS.

At 66 days post inoculation it was observed that two from five of the PVX/SP6A inoculated plants had flowered (Fig 3.18). None of the PVX/FT, PVX/mFT and control plants had flowered at this time. At 90 days post inoculation all of the PVX/SP6A plants had flowered while one from five of the PVX/mFT and control plants flowered while none of the PVX/FT plants flowered at this stage as seen in previous experiment. Interestingly when a comparison was made between the first and second flowering assay it was observed that around 90-94 days post inoculation the control plants flowered while none of the PVX/FT plants flowered. On the other hand the tomato *FT* gene *SP6A* could induce early flowering in brassica. This is interesting because the homologue of this gene in potato induces tuberisation rather than flowering.

RT- PCR test was carried out on young leaf material harvested from the mature brassica plants to detect if the virally expressed RNA transcripts were present. Results indicated that the virally expressed tomato *SP6A* RNA was detected in all the young leaves of the brassica plants except for plant 5 (Fig 3.19A). Surprisingly the RT-PCR results were negative for leaf samples harvested from PVX/FT and PVX/mFT (Fig 3.19B and C).

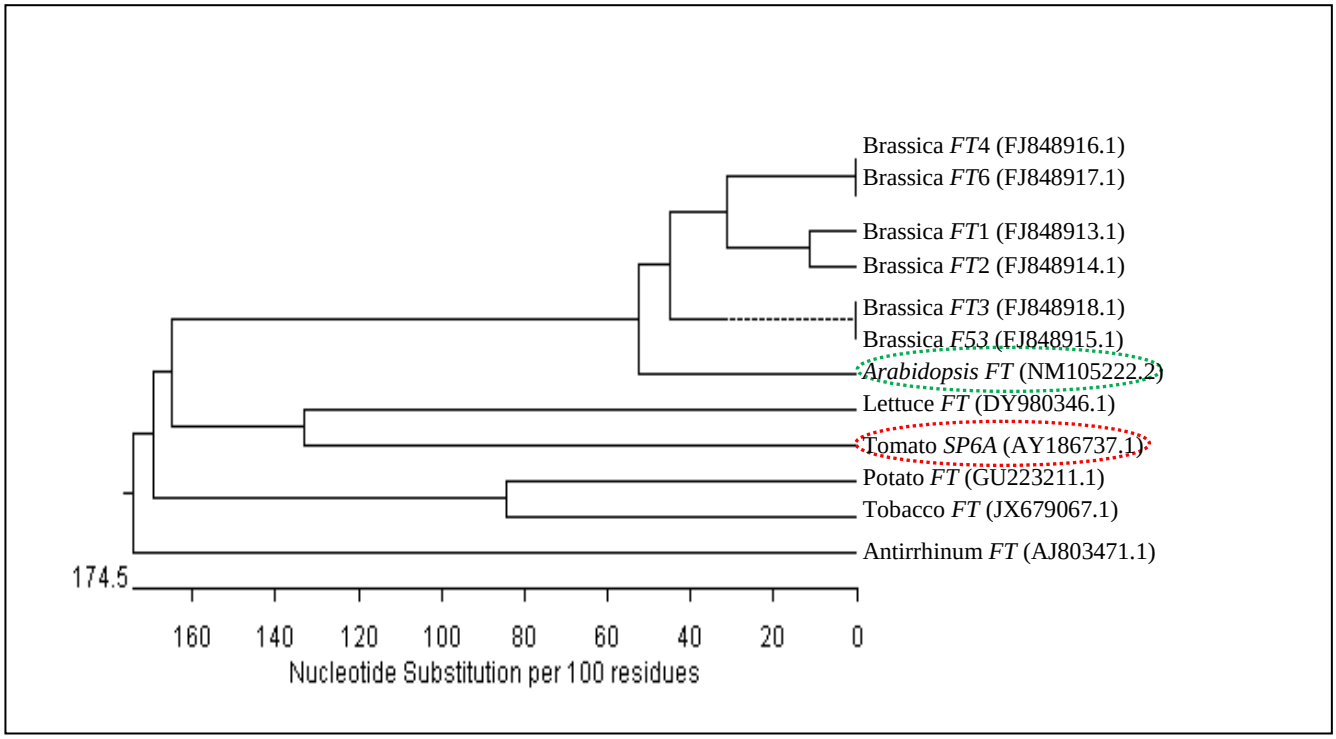


Figure 3.17 The phylogenetic relationship of *FT* orthologues among, Tomato, Potato, Tobacco Antirrhinum, *Arabidopsis*, lettuce and brassica. The evolutionary relationship was inferred using the ClustalV method. Accession numbers are stated next to the species.

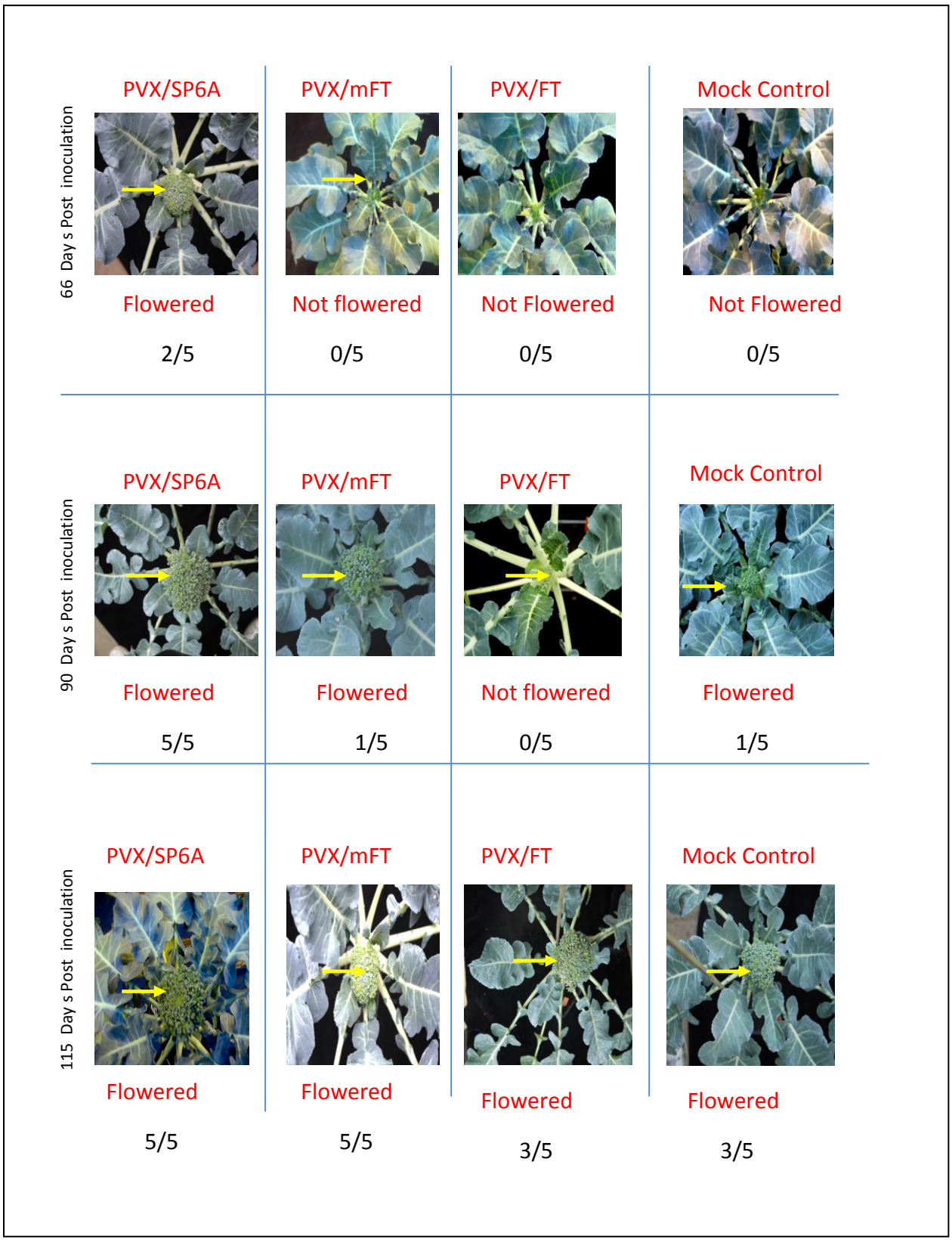
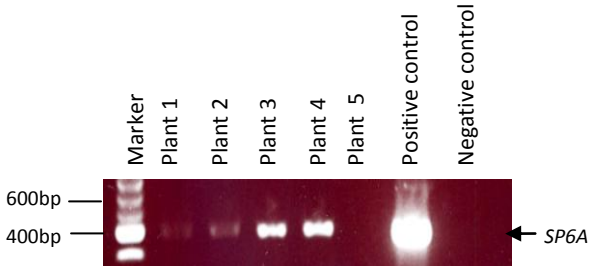
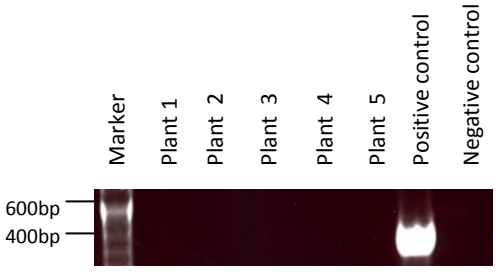


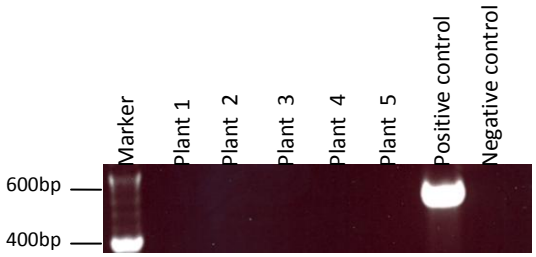
Figure 3.18 Brassica oleracea var.*italica* at 66, 90 and 115 days days post inoculation. The yellow arrows show the flower head.



A. SP6A gel



B. FT gel



C. mFT gel

Figure 3.19. RT-PCR detection of virally expressed *FT* genes in Brassica young leaf tissue samples. Positive products were detected for *SP6A* (423bp) (A). No products were detected for *FT* (750bp) and m*FT* (750bp) in (B and C). 5µl of 1kb ladder marker (invitrogen) was used. PVX specific forward primer PP82 (►) and the respective gene specific reverse (◄) primer was used. Positive control used in each lane was the respective recombinant plasmids while water (as template) was used in negative control.

Conclusion

The data presented in this chapter shows the multifunctional roles that *FT* and *FT* orthologues plays in different plant species. In Tobacco, the PVX/FT inoculated plants flowered early because the transition to the reproductive phase had been triggered by viral expression of the *Arabidopsis FT* even though they were being grown in non-inductive LD conditions. Other test plants (PVX/mFT and Mock control) remained in the vegetative phase. Viral expression of *Arabidopsis FT* in potato did not seem to have an effect on tuberisation as only one from five of the PVX/FT inoculated plants had tuberized. It would be of interest to investigate whether other *FT* orthologues would have any effect on tuberization in potato e.g the Onion *FT* gene (*AcFT1*) which has a role in bulbing could be explored. In Brassica, the viral expression of *Arabidopsis FT* caused delayed flowering. This occurrence could be as a result of VIGS due to the relatively high similarities in sequence of the *Arabidopsis FT* and endogenous Brassica *FT* as shown in the comparative sequence alignment in appendix II. Interestingly viral expression of tomato *FT* (*SP6A*) which was less similar in sequence to the endogenous brassica *FT* trigged early flowering in Brassica. On the other hand it would be interesting to investigate the effect of viral expression of potato *FT*, tobacco *FT* and Antirrhinum *FT* on flowering in brassica as these *FT* sequences are even less similar in percentage homology compared to the tomato *FT* as shown in appendix II.

In Tomato, the viral expression of *Arabidopsis FT*, mutant *FT* (FTC4) and *FT* orthologues; *SP2I* and *SP5G* caused increased seed production. The expression of these genes also had an effect on branching in tomato which could suggest the genes have possible interaction(s) with hormonal regulation in plants. The exact mechanism of action of these genes in the control of seed production and lateral side shoot development is unclear.

Chapter 4

The Expression and Functionality of Tagged *Flowering Locus T*

Chapter 4: The Expression and Functionality of Tagged *FT*

4.1 Introduction

In photoperiodic plants changes in day length are perceived by photoreceptors in the leaves. Under inducing conditions a mobile signal is produced and transported through the phloem translocation stem to the SAM where it interacts with FD to initiate flowering (Corbesier *et al*., 2006). FD belongs to the basic leucine zipper (bZIP) transcription factor family which is responsible for sequence-specific DNA binding. The leucine zipper has a super coiled structure and is involved in the homo- and /or hetero-dimerization of proteins (Landschulz *et al*., 1988).

Various experiments have shown that *FD* interacts with *FT* to trigger flowering. The requirement of *FD* by *FT* to induce flowering was confirmed by site-specific mutations in the *FD* gene. Mutant *fd* lines were reported to have a delay in both up-regulation of *AP1* expression, and early flowering phenotype, caused by *FT* overexpression (Blazquez 2005). In 2005, Wigge *et al* showed that ectopic expression of *FD* caused the up-regulation of *AP1* expression in leaves only when they were subjected to treatments that increased *FT* expression. The physical interaction between FT and FD proteins was confirmed by yeast two-hybrid assays although little is known about the DNA binding domain of FT (Blazquez 2005).

In the quest to study tissue-specific *FT* expression, Takada *et al.* (2003) fused the *FT* gene promoter upstream of the *GUS* reporter gene and transformed it into *A. thaliana*. Histological analysis of GUS staining revealed that the expression of *GUS* was detected in the vasculature tissue but not in the meristem. Other studies were carried out by Li *et al*.,(2009) using the *GFP* reporter gene. The virus expression construct (PVX/GFP) was shown to infect *N.benthamiana* and systemic infection was established and detected at 9 dpi. However, no viral expressed GFP was detected in the SAM (Li *et al*., 2009). It was observed that when GFP was tagged unto the C-terminal of *FT* (PVX/FT-GFP), systemic infection was established in inoculated plant but also that the PVX/FT-GFP transcripts were detected in the SAM. This confirmed that *FT* was capable of long distance travel from the vasculature into the SAM and enabled PVX to overcome meristem exclusion and get into the SAM.

In this chapter the functionality of polyhistidine and FLAG tagged *Arabidopsis* FT at the N- and C- terminal would be investigated. Studies would be carried out to test the effectiveness of the tagged FT in inducing flowering in tobacco under non-inductive LD condition. The other aim of this chapter is to investigate the *in vivo* distribution of the tagged *Arabidopsis* FT protein in different tissue material harvested from the inoculated plant.

4.2 Experimental Results

4.2.1 Expression of fused *Arabidopsis FT* in Maryland Mammoth tobacco under non-inductive LD condition

Young tobacco plants were maintained and inoculated at the 5-6 leaf stage. Four plants were used for each construct. Inoculation of plants was carried out with sap inoculum. The following constructs were designed and used in this experiment; PVX/FT-HIS, PVX/FT, PVX/FT-FLAG and PVX/HIS-FT (Fig 4.1). PVX/FLAG-FT was not used in this experiment because it contained a point mutation within the *FT* sequence.

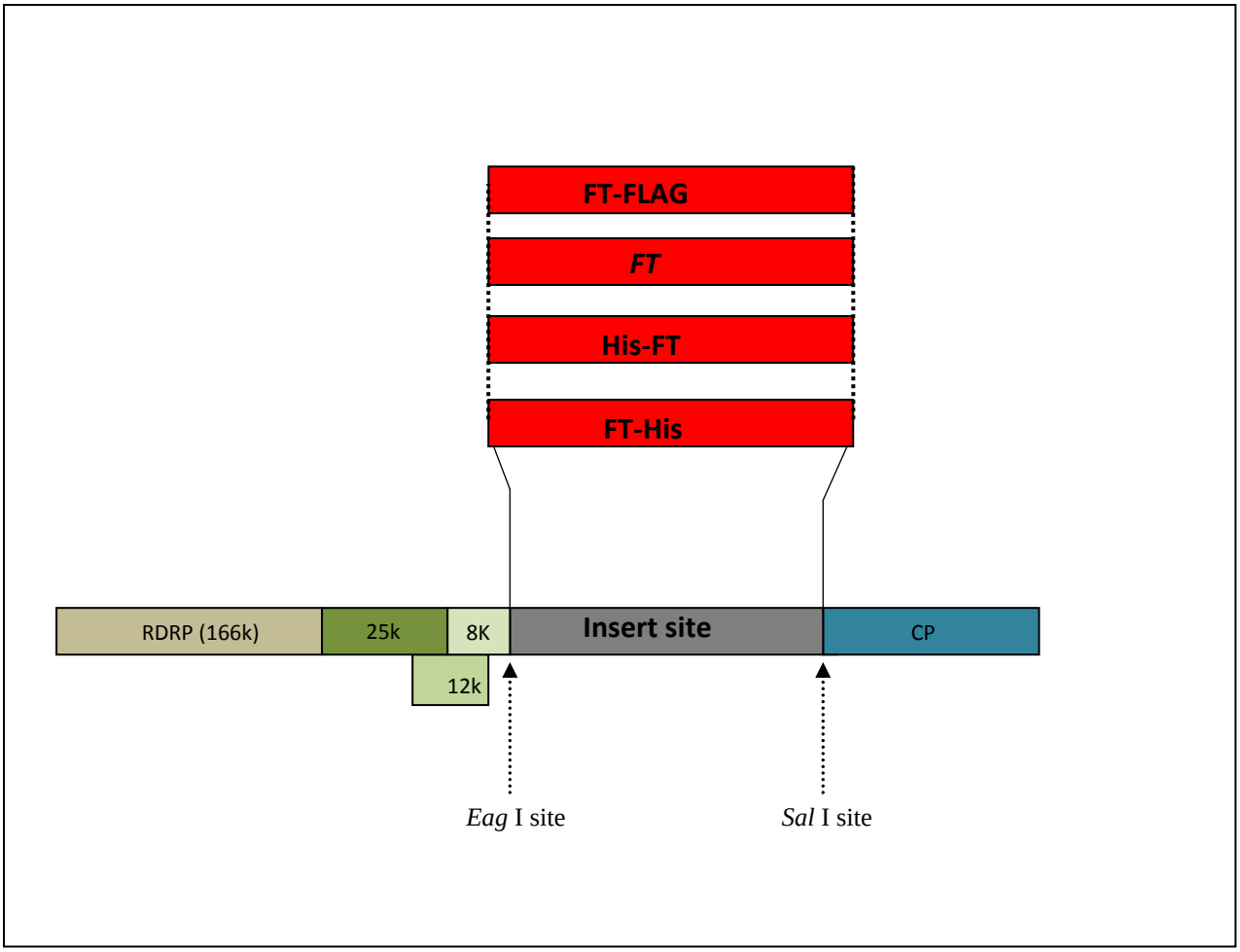


Figure 4.1 A schematic representation of the expression constructs used in the experiment.. Recombinant plasmids were linearised with *SpeI* prior to *in vitro* transcription. The RNA dependent RNA polymerase (RDRP) (166K) is involved in promoting PVX replication while the Coat protein (CP) is involved in cell to cell movement and systemic spread in host plant. WT *Flowering locus T* (*FT*), WT *Flowering locus T* (FT) tagged with polyhistidine (His) at both C- and N- terminal and C –terminal FLAG tagged FT respectively were cloned into the *Eag I* and *SaI 1* restriction sites.

At 7 days post inoculation, visible viral infection symptoms were observed on the young leaves of all the PVX/FT, PVX/His-FT, PVX/FT-His and PVX/FT-FLAG inoculated plants but not mock-inoculated plants. The symptoms included the appearance of yellow patches (chlorosis) on the leaves (Fig 4.2; Red arrows) and the leaves also appeared shrivelled around

the edges. All control plants appeared healthy as they were not infected with the virus or by other pathogens.

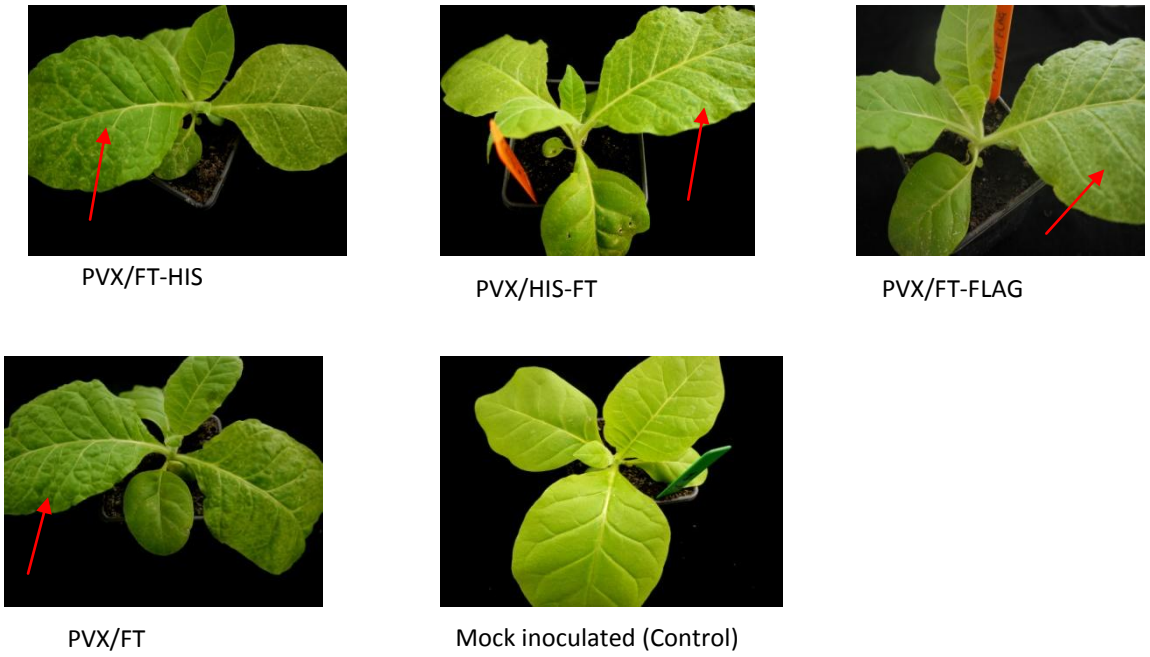


Figure 4.2. Young *N. tabacum* Maryland Mammoth plants exhibiting viral infection symptoms at 9 days post inoculation. The red arrow indicates the chlorotic lesions seen across the surface of a systemic leaf.

At 21 days post inoculation it was observed that all the PVX/FT and PVX/FT-His inoculated plants had started to bolt (Figure 4.3). Three out of four of the PVX/FT-FLAG inoculated plants bolted 6 days later. The last PVX/FT-FLAG plant died and was discarded. Bolting was characterized by a distinct increase in stem length. The stem length of the plants inoculated with PVX/His-FT and the mock- inoculated plants remained short. This is because these plants remained in their vegetative stage, which is typically characterised by a relatively slow stem growth and an increase in leaf surface area, in non-inductive LD conditions. RT-PCR test was carried out on young systemic leaves harvested from all the plants that were inoculated with PVX/FT, PVX/FT-His, PVX/FT-FLAG and PVX/His-FT. The test confirmed that the PVX RNA transcripts were present in systemic leaf tissue from all inoculated plants (Fig 4.4).

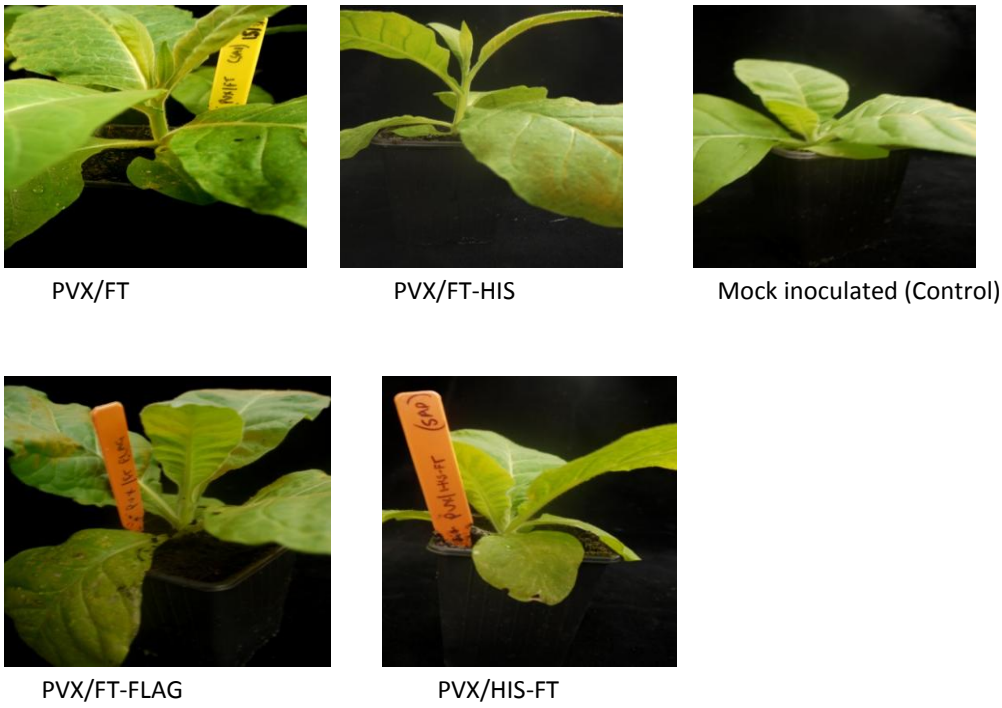
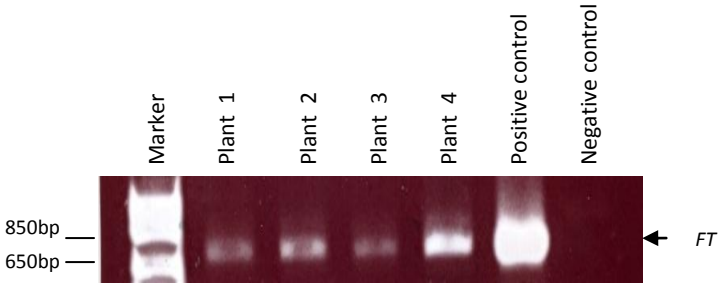
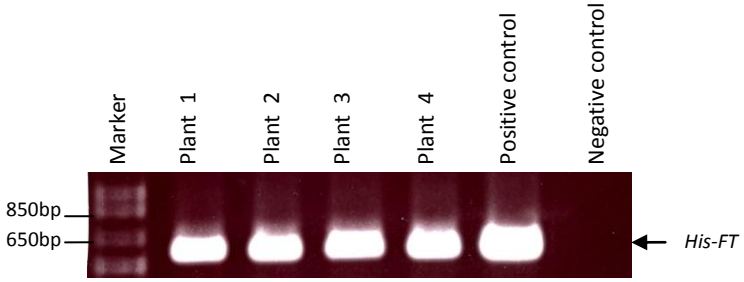


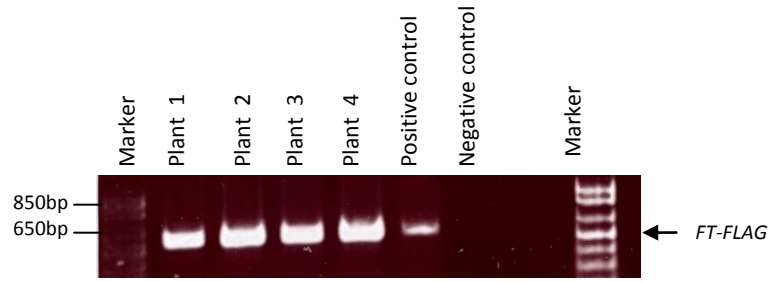
Figure 4.3 Young *N. tabacum* Maryland Mammoth plants at 21 days post inoculation. PVX/FT and PVX/FT-His inoculated plants had started to bolt while PVX/FT-FLAG bolted 5 days later. Mock control and PVX/His-FT plants remained in the vegetative stage.



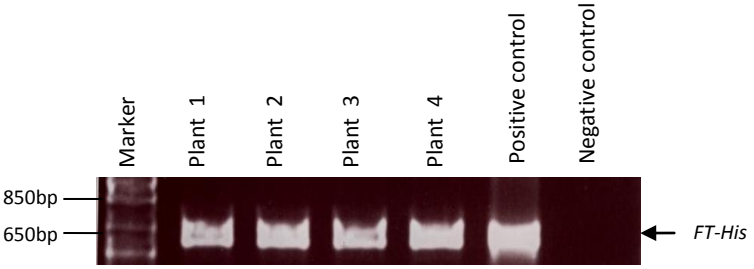
A. FT gel



B. His-FT gel



C. FT-FLAG gel



D. FT-His gel

Figure 4.4 RT-PCR detection of virally expressed *Arabidopsis FT*, *FT*-His, His-*FT* and *FT*-FLAG RNA in systemic leaf tissue samples of inoculated Maryland Mammoth tobacco plants. PVX specific forward primer PP82 (►) and the respective gene specific reverse primer was used. Positive controls used in each case was 5µl of the respective recombinant plasmids while water (as template) was used as the negative control. 5µl of 1kb plus ladder

At 48 days post inoculation it was observed that the floral buds of all the PVX/FT-His inoculated plants had opened. 4 days later all the PVX/FT inoculated plants flowered while two from three of the PVX/FT-FLAG inoculated plants flowered 23 days later, figure 4.5. The mean stem length of the PVX/FT-His, PVX/FT and PVX/FT-FLAG (mean of the two from three plants that flowered) inoculated plants at this stage was 49cm, 55cm, 35cm respectively (Fig 4.6). There was a significant difference in stem lengths between the PVX/FT, PVX/FT-His and PVX/FT-FLAG compared to Mock control (Fig 4.6).

PVX/FT-HIS PVX/FT

PVX/FT-HIS PVX/His-FT

PVX/FT-HIS PVX/FT-FLAG

PVX/FT-HIS Mock control

Figure 4.5. *N. tabacum* Maryland Mammoth plants at 52 days post inoculation. PVX/FT-His, PVX/FT and PVX/FT-FLAG inoculated plants had developed flowers or floral buds. PVX/His-FT and Mock control plants remained in the vegetative phase.

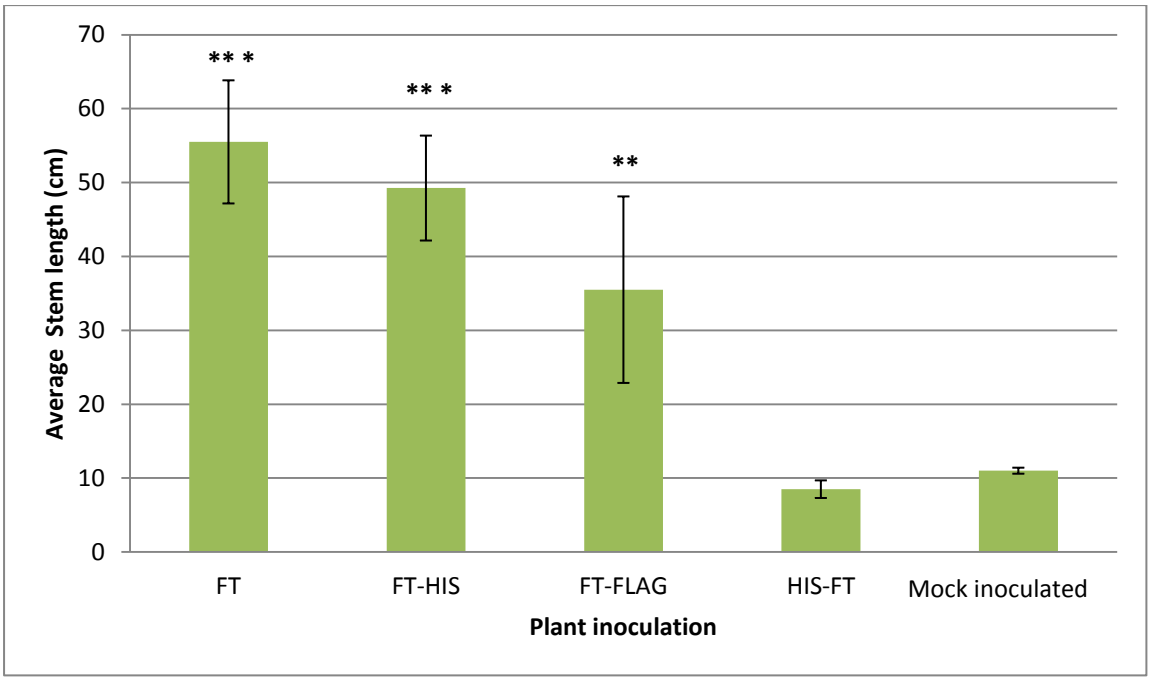


Figure 4.6 Effect of expression of tagged FT protein in tobacco. The average stem length (cm) for each test plant group 52 days post inoculation. (n=5). Error bars indicate the Standard error. P values; ***** = P < 0.05, ****** = P < 0.01, ******* = P<0.001).

Plants in the other treatments (PVX/His-FT and mock control) continued to remain in their vegetative stage which was characterised by a relatively slow stem growth. The mean stem lengths after 52dpi were 9cm and 11cm respectively (Fig 4.6). The PVX/FT and PVX/FT-His inoculated plants flowered early because the transition to the reproductive phase had been triggered by viral expression of the *Arabidopsis FT* even though they were being grown in non-inductive LD conditions.

Conclusion

The data presented in this chapter demonstrates that the virally expressed C terminal His tagged FT and WT FT triggers early flowering in tobacco plants. Delayed flowering was observed in the plants that expressed the N terminal His tagged FT and C terminal FLAG tagged FT. A plausible explanation for the delayed flowering observed in the plants that were inoculated with PVX/His-FT could be due to the hexa histidine fusion at the N terminal of FT. This could have disrupted the overall structural conformation of FT thereby hindering its biological activity by possibly preventing hetero-dimerisation with FD (as described in 4.1).

The PVX/FT-FLAG inoculated Maryland Mammoth tobacco plants flowered later than the PVX/FT and PVX/FT-His inoculated plants mostly possibly due to the size of the FLAG tag that was attached unto FT as it has been reported that protein size is a critical factor of protein movement (Crawford *et al*., 200; Wu *et al*., 2002; Wigge *et al*., 2003). FLAG tag has a molecular weight of 1012Da, while the 6x His tag has a molecular weight of 840Da. This additional size of the tag affects the function and/ or movement of the fusion protein which could explain why eventhough the *FT*-FLAG transcripts were detected in the systemic leaves bolting and floral induction occurred later compared to the WT FT and FT-His plants.

Apart from size and positioning of the tags another plausible explanation could be due to the solubility of the tags. Various tags possess different hydrophilic nature which could have a contributory effect on the overall function of the tagged protein of interest e.g Glutathione S-transferase (GST) and Thioredoxin are known to have very high hydrophilic levels and they have also been shown to enhance the solubility of expressed proteins. It could be that the FLAG tag's hydrophilic level was lower than the His tag which could possibly explain why the FT-FLAG tagged infected plants flowered later than the FT-His inoculated plants.

Another plausible explanation could also be due to the relative half- life of the tags. As proteins have different half-lives one cannot rule out the possibility of the degradation of these tags *in vivo*. The difference in the half-lives of the tags used could have a contributory effect for example a protein tag with a relatively short half-life would result in free untagged virally expressed *Arabidopsis* FT protein. These proteins would migrate to the SAM and bind with FD forming a complex which ultimately triggers flowering. This suggestion however would be only valid upon carrying out additional tests on tissue material harvested from the

young leaves and SAM of the inoculated plant in order to confirm the presence or absence of tagged/untagged FT protein.

In order to detect the fusion proteins, western blot analysis was carried out but this was unsuccessful. More work would be needed in future in order to achieve this objective. Functional tagged FT such as C-terminal tagged FT would provide the possibility of studying the localization and tracking its movement *in situ*. Other possibilities such as investigating FT's binding/interactions with other compounds could be explored. Unpublished reports have shown that FT can bind to specific cellular proteins such as 14-3-3 protein. Pull down assays which would include a selective bait protein to bind to other proteins. These proteins could be analysed in detail and research could be carried out on the corresponding genes e.g Gene knock- out could be utilised to study the biological functions or significance of the genes to FT movement and/or floral induction.

Chapter 5

Coat Protein transgenic plant complementation

Of movement deficient Virus

5.1 Introduction

In both field and glasshouses, the spread of seed-transmitted viruses occurs through different vectors including whiteflies, aphids etc. Infected seedlings from virus-infected seeds provide the primary site of infection from which further spread occurs through insect vectors or mechanical cross contamination. The majority of the seed-transmitted plant viruses spread through arthropod vectors e.g. whiteflies, aphids and beetles (Nault *et al*., 1997). In addition to insect vectors, nematodes and fungi have been reported to be other agents of virus spread (Harris *et al.,* 1981; Campbell *et al*., 1996). Insect-borne plant viruses are accountable for many losses in crops and reduction in harvest yield (Raccah *et al*., 2009). The outbreak of viral diseases caused by insect vectors has been reported in many different geographic locations. For example in the 1930's in Argentina and Brazil the citrus industry suffered drastic losses which were attributed to the aphid *Toxoptera citricidus*. This same aphid was also found in Portugal and Spain and has been threatening the Mediterranean citriculture. In the last decade, Outbreaks of *Tomato spotted wilt virus* (TSWV) have been attributed to the spread of thrips *Frankliniella occidentalis* (Raccah *et al*., 2009).

Most of the classified insect vectors are piercing-suckers and they transmit the virus either in a noncirculatory or circulatory manner. In the noncirculatory mode of transmission, the virus is carried on the lining cuticle of the insect's stylet while in the circulatory mode the virus passes through the insect's gut and then moves internally to the salivary glands and is eventually expelled during feeding to inoculate a new host plant. Transmissibility of circulatory viruses depends on proteins comprising the virus capsid e.g. the coat protein. Passage of circulative virus through the gut has been also associated with vectors' proteins. In both scenarios the virus is transmittable to healthy plants (Raccah *et al*., 2009). The most common strategy for virus-vector interaction is the noncirculatory mode of transmission. A feature of the noncirculatory mode is that several virus species can be transmitted by the same vector and also several vector species can transmit the same virus. Uzest *et al*., (2007) reported the precise location of the first receptor for non circulative virus; cauliflower mosaic virus and its insect vector. Electron microscopy revealed virus-like particles in the tip of the aphid's maxillary stylets. *In vitro* assays were also utilized to visualise the interaction between the dissected aphid stylets and cauliflower mosaic virus protein P2 which is involved in the virus-vector interaction. P2 GFP fusion proteins revealed the protein was exclusively

located in the bottom-bed of the salivary duct of the insect. A point mutation within P2 was also reported to correlate to impaired stylet binding (Uzest *et al.,* 2007).

The transmissibility of viruses essentially depends on the coat protein (CP) (Atreya *et al.,* 1993). The CP is encoded by an ORF located in the genome of potyviruses and potexvirus. The CP is an important structural protein involved in cell-to-cell and systemic movement of the virus (Gilbertson *et al*., 1996; Wellink *et al*., 1989; Chapman *et al*., 1992; Van der Vossen *et al*.,1994; Dolja *et al*., 1994).

In 1992, Chapman *et al* designed a PVX construct which had the CP coding sequence replaced with a *GUS* gene. It was reported that the viral construct was incapable of establishing systemic symptoms on inoculated *Nicotiana tabacum* (Chapman *et al*., 1992). This demonstrated that the CP was required for systemic movement of PVX virus. The role of the DAG motif of CP in aphid transmission was also confirmed for a Non-aphid transmittable (NAT) strain of *Zucchini yellow mosaic virus* (ZYMV). An amino acid change of Threonine to Alanine (DTG to DAG) resulted in a restoration of transmissibility (Atreya *et al*., 1995). Electron- microscopic studies also provided evidence to confirm that the DAG motif was involved in retaining the virus in the aphid's mouth parts. The mechanism was reported to involve the interaction of DAG with a helper component (HC) which was confirmed by protein-blotting overlay technique. The involvement of HC in retention of virus in the stylet was demonstrated by comparing aphid's fed on mixtures of transmissible TEV virions and TuMV HC with those fed on non-functional HC (Harris *et al*., 2001).

Site-directed mutagenesis of the CP gene of tobacco vein mottling virus (TVMV) showed that most substitutions and deletions within the gene caused a loss, or drastic reduction, of transmissibility (Atreya *et al*., 1995). It was also reported that aphids were unable to transmit the resultant hybrid virus which had the TVMV-NAT (Non-aphid transmittable) coat protein, although the concentration and infectivity of the hybrid virus in the source plants were similar to those of TVMV-AT (Aphid transmittable). In an isolate of tobacco etch virus (TEV) which contained two consecutive DAG motifs separated by a single alanine, transmissibility was reported to be abolished by mutations affecting the first motif while mutations in the second motif had little effect (Lopez-moya *et al*., 1999). In *cucumber mosaic virus* (CMV) which is normally a poorly transmitted virus it was shown that three amino acid changes in CP affected the transmission of CMV by *Aphis gossypii* (Perry *et al.,* 2001). Further investigation

by the same research group revealed that the transmissibility of CMV by *Myzus persicae* required two mutations within the 25th and 214th position of CP. Conserved motifs other than DAG have also been identified for example in pea mosaic virus (PMV) the DAS motif (Johansen *et al.,* 1996), and in peanut mottle virus (PeMoV) - the DAA motif (Flasinski *et al*., 1998).

As the CP is involved in cell-to-cell or systemic movement of PVX which is important for establishing infection, PVX could be utilized as a valuable tool for gene study and analysis of RNA movement. For example, movement deficient PVX recombinant virus containing GFP coding sequence was used in investigating RNA movement (Bauclombe *et al*., 1995). In addition, recombinant PVX vectors are viable tools for the production of pharmaceutical proteins such as vaccines (Scholthof *et al*., 1996). In the agricultural and horticulture sector, various strategies have been employed to combat virus spread in fields. In this chapter, transgenic technology will be utilised to promote movement deficient viruses whose localisation is limited to just the inoculated plant thereby reducing the likelihood of cross contamination to neighbouring plants. We hypothesise that an intact CP transgenic tobacco plant would rescue a CP mutated movement deficient recombinant PVX construct.

In this chapter, I aim to investigate whether *FT* and /or viral mRNA enters the germline in inoculated plants and is transmitted through to the seeds. I also investigate whether CP producing transgenic plants can compensate for the lack of CP in a CP mutated movement deficient recombinant PVX construct expressing the *Arabidopsis FT* RNA/protein: PVX/FTΔCP (kindly provided by Prof Yiguo Hong). I also aim to investigate if this construct is capable of long distance movement and also test its effectiveness in inducing early flowering.

5.2 Materials and Methods

5.2.1 Gateway cloning of CP gene into binary vector and transformation of *Agrobacterium tumefaciens*

The coat protein (CP) gene was amplified from PVX plasmid using AttB1CPF and AttB2 CPR primers. CP DNA was amplified using the 2 –STEP thermal profile which was 95^{o}C 2mins, 95^{o}C 20sec, 55^{o}C 10sec, 70^{o}C 10mins at 4 cycles followed by 95^{o}C 20sec, 65^{o}C 10sec, 70^{o}C 1min and 70^{o}C for 4mins at 20cycles. The resulting PCR product was purified and verified by direct sequencing using the AttB1CPF and AttB2CPR primers. Purified CP DNA was then incorporated into the Gateway® pDONR 207™ Vector (Invitrogen Ltd., USA) using the Gateway® BP Clonase® II enzyme mix (Invitrogen, Cat No. 11789-020) following the manufacturer's instructions. Recombinant pDONR 207™ was later transformed into EC100 electrocompetent cells using an electroporator (BioRad) (see 2.2.12 **)**.Transformed cells were spread on a LB agar plate containing 25µg/ml Gentamicin and positive single colonies were screened, sequenced and analysed before proceeding to the next Gateway cloning step.

In the LR reaction step (Fig 1.5), the recombinant pDONR 207™ /CP and Binary vector PB2GW7 (Invitrogen Ltd., USA) were added into an Eppendorf tube containing Gateway® LR Clonase II enzyme mix (Invitrogen, Cat. No. 11791-043 USA). Recombinant PB2GW7/CP was then transformed into EC100 cells. Transformed cells were poured onto LB agar plates containing 100µg/ml Spectinomycin. Positive single colonies were screened, sequenced and analysed. The recombinant binary vector harbouring the *CP* gene (Fig 5.2) was then transformed into *Agrobacterium tumefaciens* strain AGC58PGV3101 (see 2.2.13). Single colonies identified were screened and analysed (see section 2.2.15).

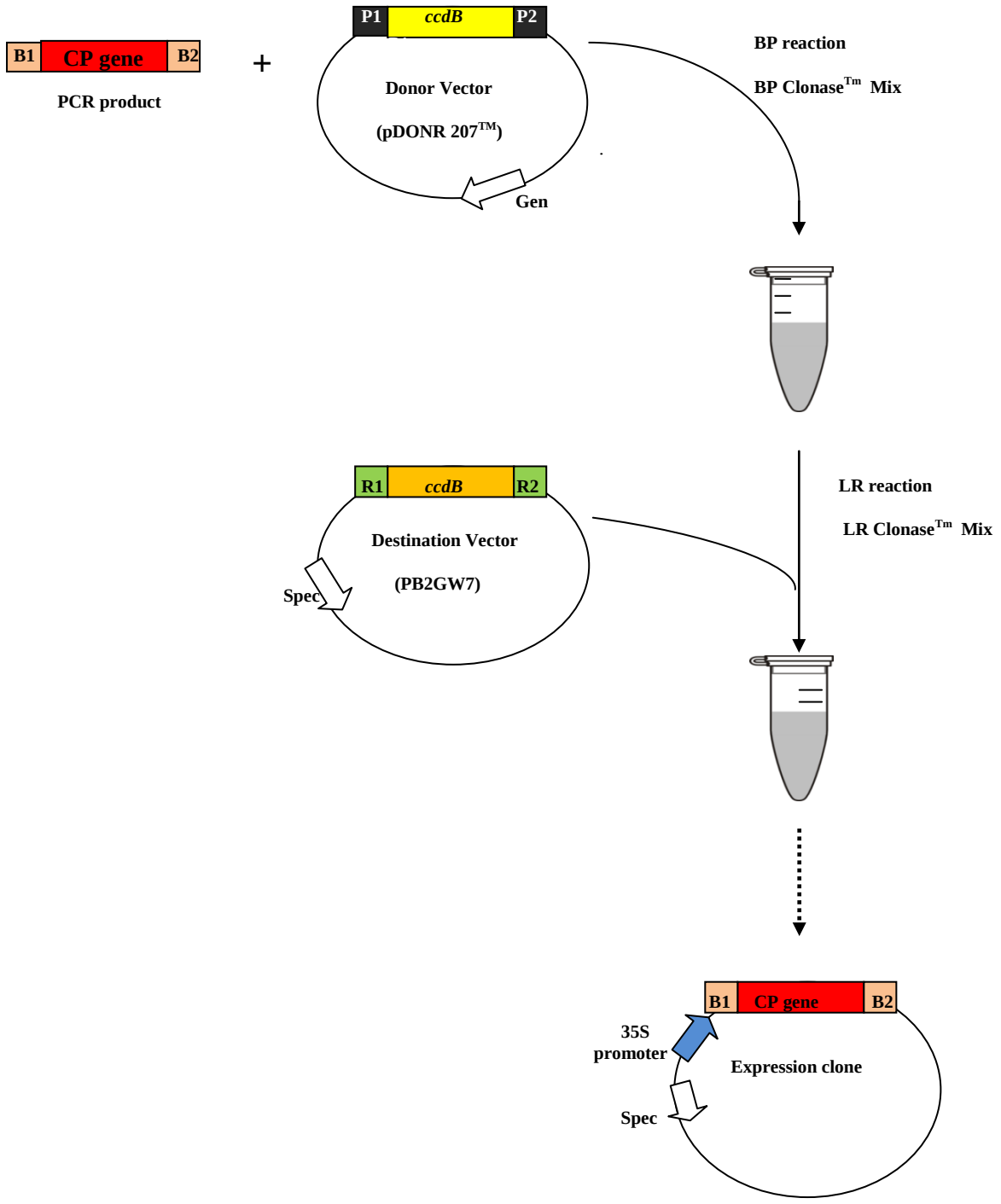


Fig. 5.1 An overview of the cloning of CP into 35S expression vector via Gateway cloning™ .

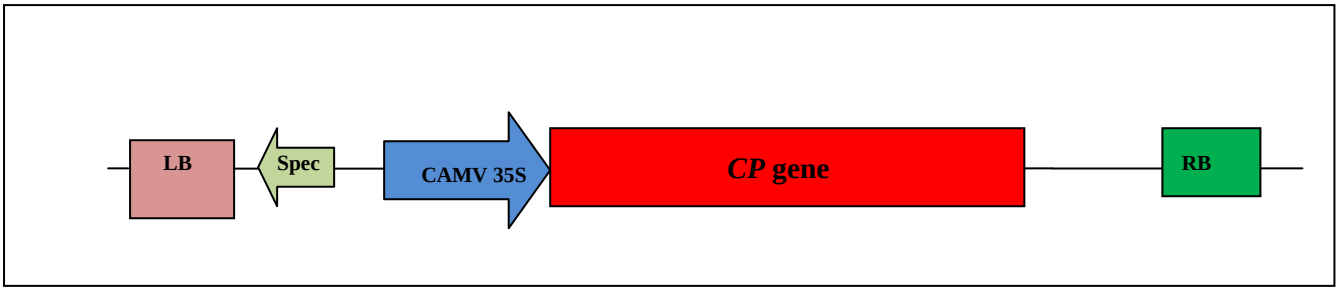


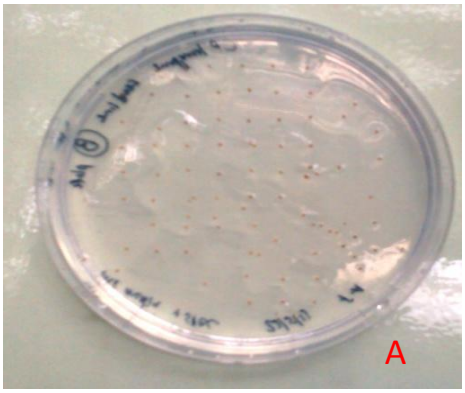
Figure 5.2 Gene construct: PB2GW-35S-*CP* used for *Agrobacterium*–mediated transformation of tobacco.

5.2.2 Tobacco transformation

Tobacco transformation was carried out as described in section 2.2.14. There were no changes to experimental conditions or regents.

5.2.3 Isolation of homozygous plants

100 seeds were harvested from four T_2 plants and sown on solid MS media that contained 100µg/ml Spectinomycin. Young seedlings were observed and scored on the basis of the leaf pigmentation; healthy green or pale white. 100% green leaf *N. tabacum* seedlings (Fig 5.3B) were transplanted into M2 soil (Levingtons) (Fig 5.3C) and used for subsequent experiments.



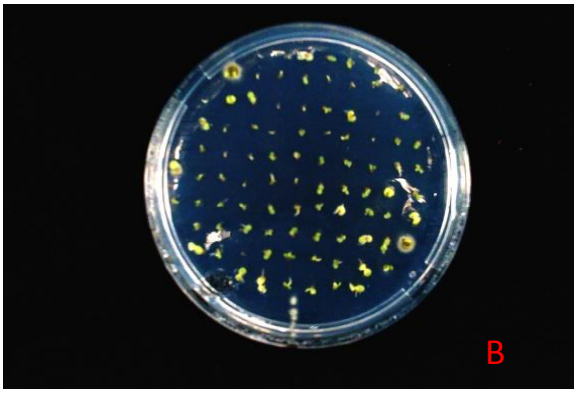


Figure 5.3. Tobacco homozygosity test A. T_2 plant's seeds on solid MS media**. B.** Young green leaf CP transgenic MM seedlings. **C**. Young CP-MM plant transplanted into soil.

5.3 Results and Discussion

5.3.1 Does the virally- expressed *Arabidopsis FT* and coat protein RNA get transmitted into the germline/seeds of inoculated plants?

In order to investigate if the virally- expressed *Arabidopsis FT* and coat protein RNA get transmitted into the germline/seeds of inoculated plants RT-PCR was carried out on young seedlings that were from seeds of PVX/FT inoculated tobacco (*Nicotiana tabacum)* and tomato *(Lycopersicon esculentum)* plants. The results indicated that neither of the virally-expressed *Arabidopsis FT* or coat protein RNA was detectable in seed material and therefore not transmitted into the germline of inoculated plants (Fig 5.4).

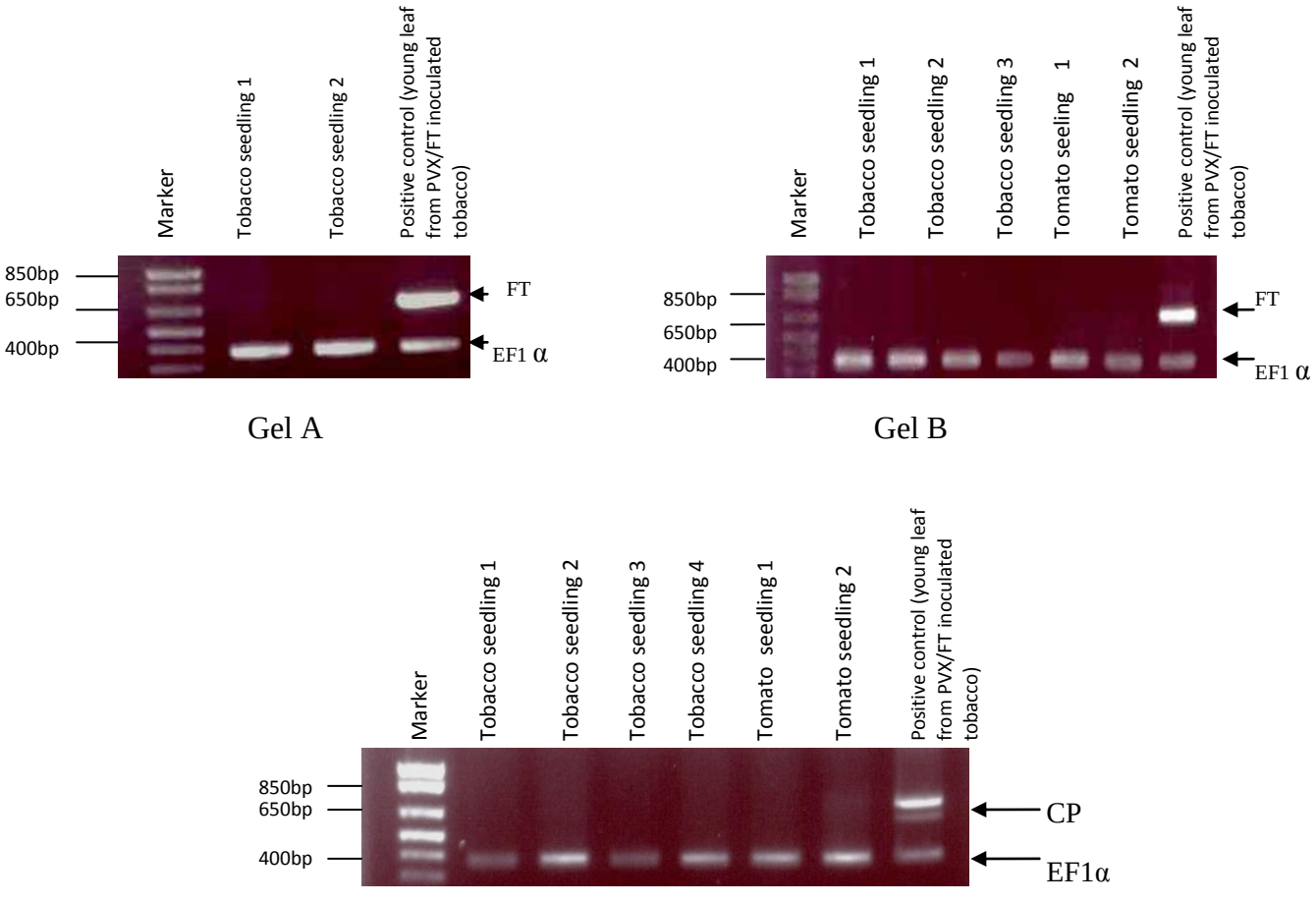


Figure 5.4 RT-PCR detection of virally expressed *Arabidopsis FT* and CP RNA in systemic leaf tissue samples of seedlings grown from seed of PVX/FT inoculated Maryland Mammoth tobacco and Ailsa Craig tomato plants. 5µl of 1kb plus ladder marker (invitrogen) was used. PVX specific forward primer PP82 (►) and gene specific reverse primer PP356 (◄), TCPF and TCPR for CP, EF1F and EF1R for House keeping gene were used. Positive control used in each lane was systemic leaf tissue material harvested from PVX/FT infected plant. **Gel A and B** represents young systemic leaf samples obtained from seedlings of PVX/FT inoculated tobacco and tomato plants. Lane 1 contains 5ul of 1kb ladder marker (Invitrogen), both gels show negative products for *Arabidopsis FT* (750bp) detection and positive PCR products for house keeping gene (Elongation factor 1 α) (380bp). Gel C represents young systemic leaf samples obtained from seedlings of PVX/FT inoculated tobacco and tomato plants. Lane 1 contains 5ul of 1kb ladder marker (Invitrogen), both gels show negative products for CP (714bp) detection and positive PCR products for house keeping gene (Elongation factor 1 α). Positive control used in each lane was systemic leaf tissue material harvested from PVX/FT infected plant.

Further investigation was carried out on the harvested seeds in order to check if the matured plants would be induced to flower early. The follow up test was carried out as an additional confirmation that the viral RNA was not transmitted into the seeds of inoculated plants. It was observed that all five plants of both control (mock) and the test plants bolted on the same day at 70 days post germination (Fig 5.5). Both plant groups also had similar mean stem length and leaf number. The control (mock) plants had a mean stem length/leaf number of 72.8cm / 29 while the test plant group had a mean stem length/leaf number of 72.6cm / 29 (Fig 5.6). As expected there was no difference in flowering time consistent with the observation that the target virally expressed RNA was not transmitted into the seeds of inoculated plants.

This proved to be an important result due to the fact that we do not want the virally expressed RNA present in the seeds as it would hinder the technology from being used as a viable tool in breeding programmes. In addition, from an environmental biosafety point of view the lack of seed transmission of PVX means seeds produced by the plants would be virus free. This provides an added value to this viral expression system.

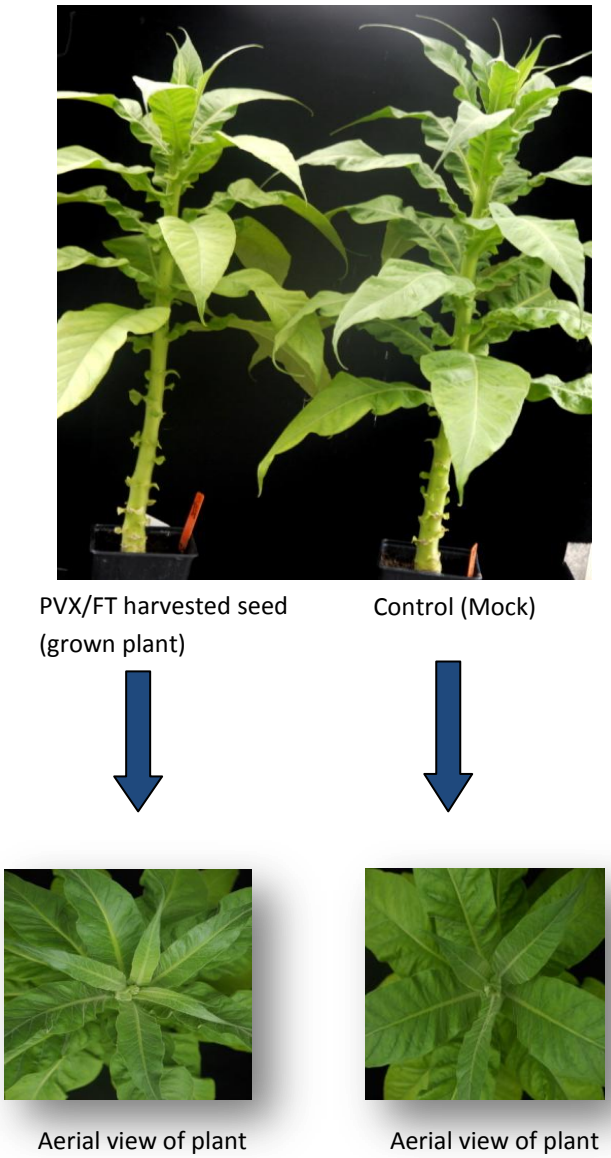
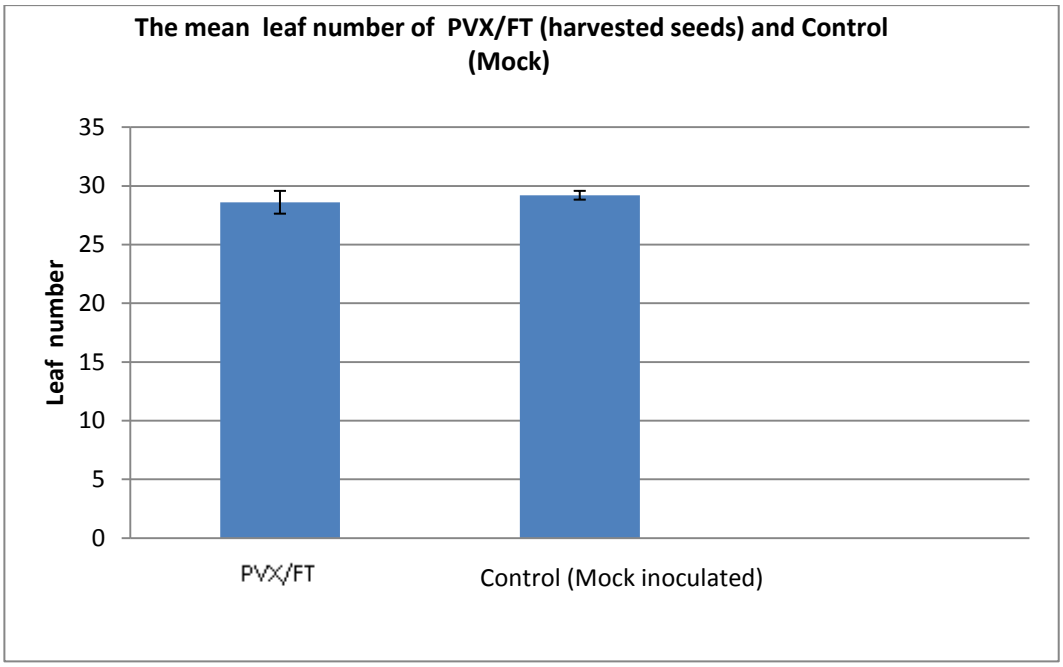


Figure 5.5 70 days post germination. PVX/FT harvested seed (grown plant) and control (mock) plant in the vegetative phase at this stage.

A



B

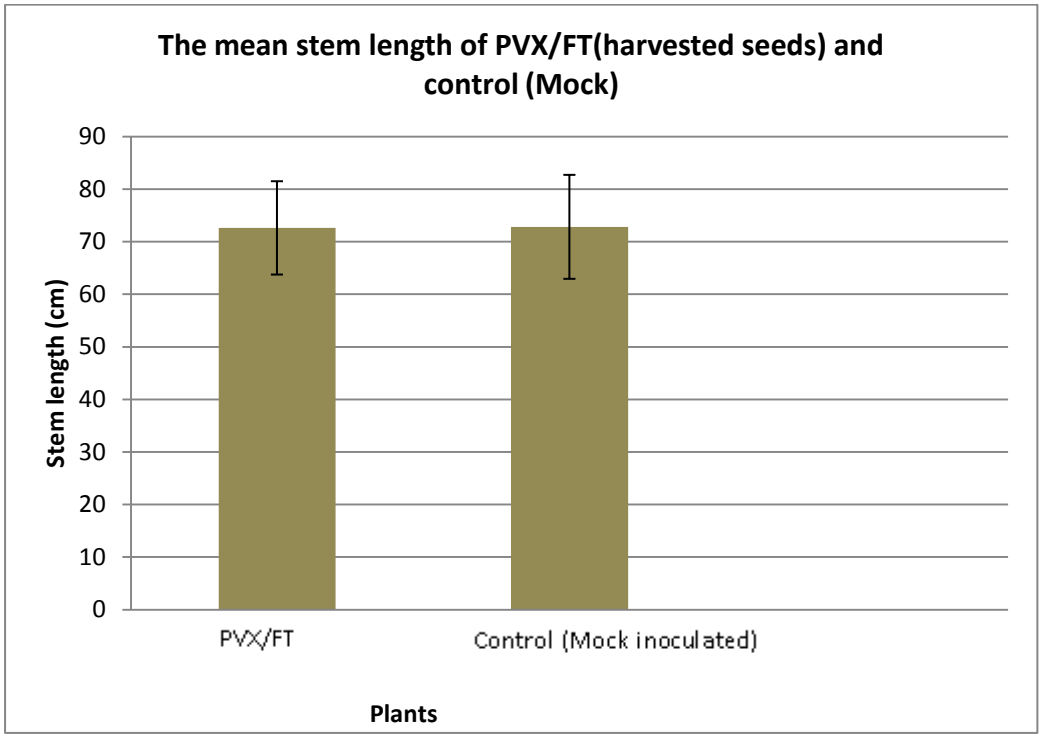


Figure 5.6 A. Shows the mean leaf number of PVX/FT harvested seed (grown plant) and control (mock) plant **B**. Shows the mean stem length of PVX/FT harvested seed (grown plant) and control (mock) plant.(n=5). Error bars indicate the standard error.

5.3.2 Expression of *Arabidopsis FT* and PVX/FTΔCP in CP transgenic Maryland Mammoth tobacco (CP-MM) under non-inductive LD condition

A preliminary experiment was carried out with two young CP-MM plants that were maintained and inoculated at the 5-6 leaf stage. Test was carried out on harvested young leaf material from both CP-MM plants in order to confirm the expression of CP transgene. RT-PCR result showed that the transgene was indeed being expressed in both transgenic plants (Fig 5.7)

Inoculation of plants was carried out with *in vitro* transcribed RNA of PVX/FT and PVX/FTΔCP respectively. It was observed that at 9 days post inoculation, visible viral infection symptoms were observed on the young leaves of the PVX/FT and PVX/FTΔCP inoculated plants although the symptoms on the PVX/ FTΔCP inoculated plants were much milder than the PVX/FT inoculated plants. This provided an indication that the host plants had been successfully infected. The symptoms included the appearance of yellow patches (chlorosis) on the leaves.

At 21 days post inoculation it was observed that the PVX/FT inoculated plant had started to bolt and the stem length at this stage was 12cm (Fig 5.8). At this stage, the PVX/FTΔCP inoculated plant still remained in the vegetative phase with a stem length of 2cm (Fig 5.8). RT-PCR test was carried out on young systemic leaves harvested from the test plants that were inoculated with PVX/FT and PVX/FTΔCP. Test confirmed that the PVX RNA transcripts were present in systemic leaf tissue from both the inoculated plants but apparently more in the PVX/FT than PVX/FT ΔCP (Fig 5.9).

At 65 days post inoculation it was observed that the PVX/FT inoculated plant had flowered. The PVX/FTΔCP inoculated plant continued to remain in the vegetative stage (Fig 5.10). As expected, the PVX/FT inoculated plants flowered early because the transition to the reproductive phase had been triggered by viral expression of the *Arabidopsis FT* even though they were being grown in non-inductive LD conditions. Surprisingly the PVX/FTΔCP inoculated plant did not flower. A possible explanation for this could be due to the low concentration of RNA transcripts that was used to inoculate the plants. In order to be certain of the results obtained, further experiments were necessary.

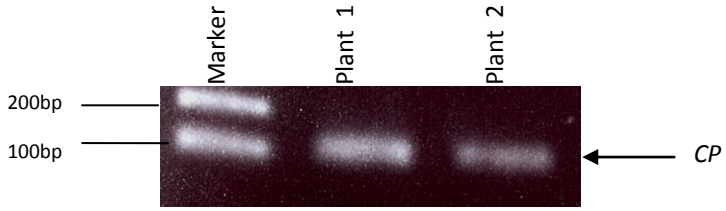


Figure 5.7 RT-PCR detection of *CP* transgene in young leaf tissue samples of CP-MM. tobacco plants. 5µl of 1kb plus ladder marker (invitrogen) was used. CP specific primer CPsF (▶) and CPsR reverse primer was used.

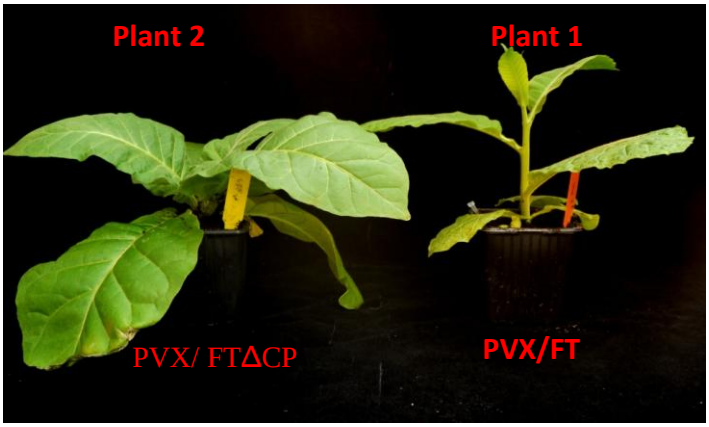


Figure 5.8 21 days post inoculation of CP-MM plants. PVX/FT inoculated plant had bolted while PVX/ FTΔCP inoculated plant remained in the vegetative phase.

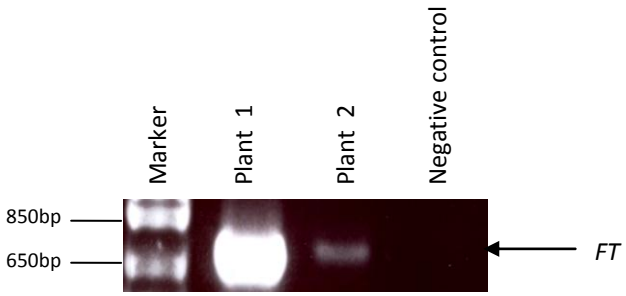


Figure 5.9 RT-PCR detection of virally expressed *Arabidopsis FT* RNA in systemic leaf tissue samples of inoculated CP-MM. tobacco plants. 5µl of 1kb plus ladder marker (invitrogen) was used. PVX specific forward primer PP82 (▶) and gene specific reverse primer (PP356) was used.

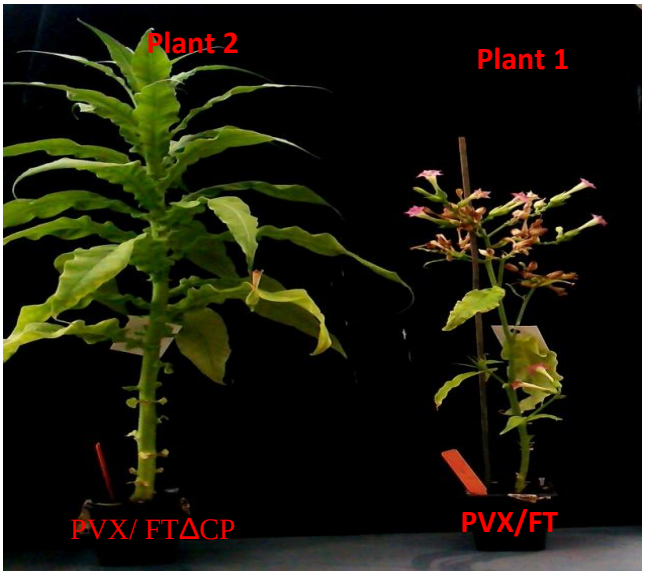


Figure 5.10 65 days post inoculation of CP-MM plants. PVX/FT inoculated plant flowered while the PVX/ FTΔCP inoculated plant remained in the vegetative phase.

A repeat experiment was carried out with 5 plants per test group. In this experiment both *in vitro* transcribed RNA and sap were used as a source of inoculant as opposed to the previous preliminary experiment in which only *in vitro* transcribed RNA was used. Sap inoculum was used because it would contain a much higher virus titre than *in vitro* transcribed RNA. The plants were inoculated at the 5-6 leaf stage. It was observed that at 9 days post inoculation, visible viral infection symptoms were seen on the young leaves of all the PVX/FT inoculated plants (Fig 5.11 yellow arrow). No visible viral infection symptom was observed in the *in vitro* transcribed and sap PVX/FTΔCP inoculated plants. All control plants appeared healthy as they were not infected with the virus or by other pathogens.

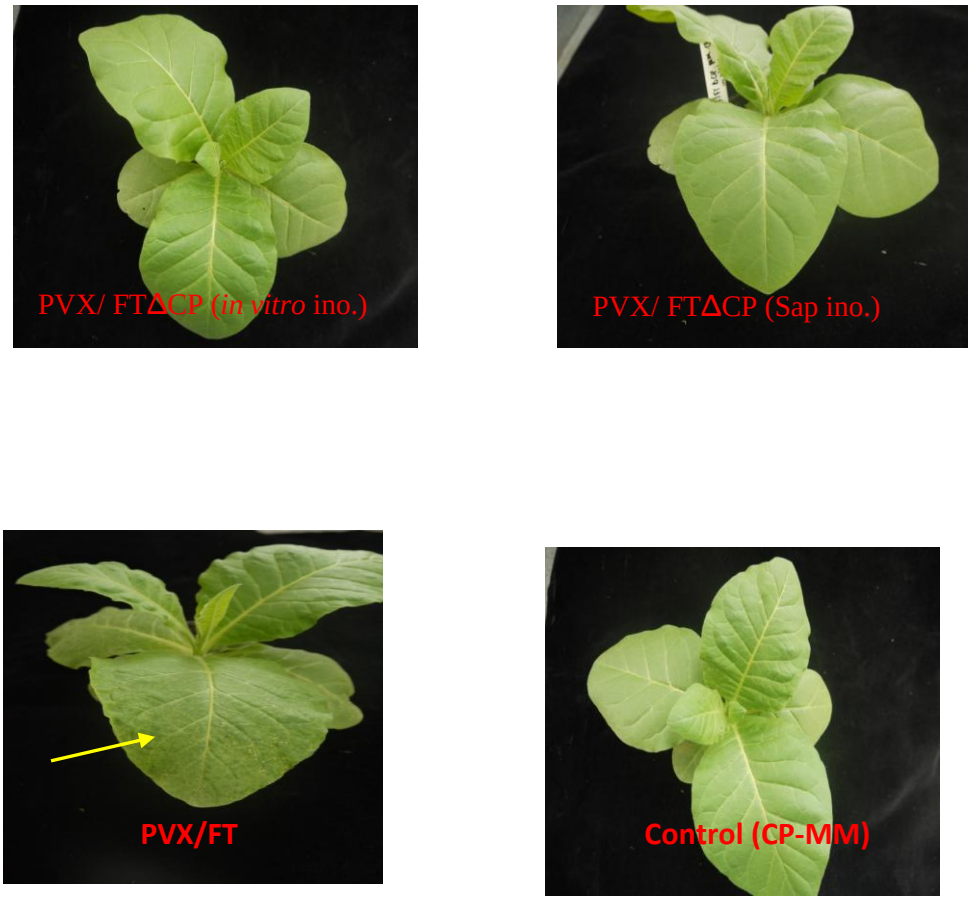


Figure 5.11 9 days post inoculation of CP- MM plants. PVX/FT inoculated plants showing viral infection symptoms while no visible viral infection symptom was observed on the control (CP-MM), PVX/ FTΔCP sap and *in vitro* RNA inoculated plants. The yellow arrow indicates the chlorotic lesions spread across the surface of a systemic leaf.

At 18 days post inoculation it was observed that all of the PVX/FT inoculated plants had started to bolt and the mean stem length at this stage was 6cm (Fig 5.12). At this stage, all the PVX/FTΔCP sap and *in vitro* RNA inoculated and control (CP-MM) plants still remained in the vegetative phase. The mean stem lengths were 3cm, 2cm and 2cm respectively. RT-PCR test was carried out on young systemic leaves harvested from all the test plants that were inoculated with PVX/FT and PVX/FTΔCP. The test confirmed that the *PVX* RNA transcripts were present in systemic leaf tissue of all the PVX/FT inoculated plant. One out of five of the PVX/FTΔCP *in vitro* RNA inoculated sample was positive (Fig 5.13 Gel B, plant 1) while no product was detected in the samples harvested from the PVX/FTΔCP sap inoculated plants (Fig 5.13 Gel C). At 40 days post inoculation it was observed that all of the PVX/FT inoculated plants had flowered (Fig 5.14). PVX/FTΔCP sap, and *in vitro* RNA inoculated,

and control (CP-MM) plants still remained in the vegetative phase including the *in vitro* RNA inoculated plant 1 that tested positive for target RNA expression.

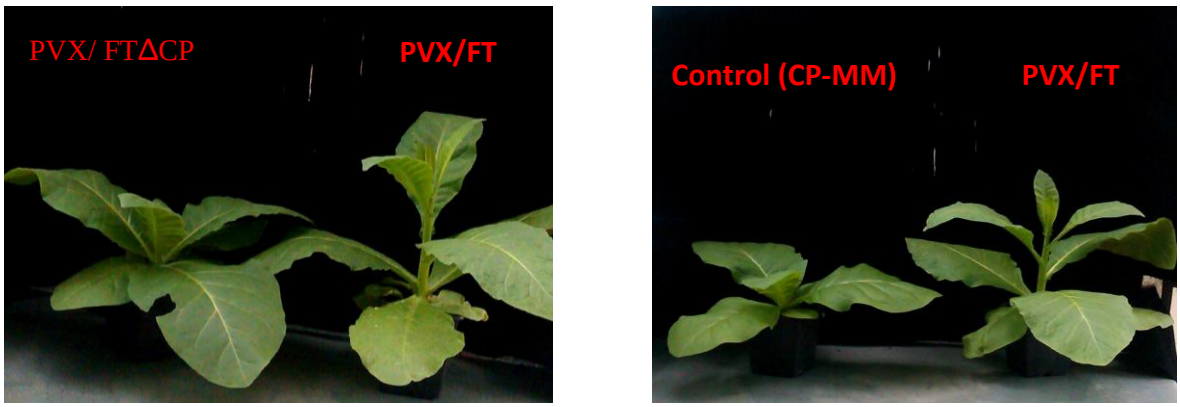


Figure 5.12 18 days post inoculation of CP. MM plants. PVX/FT inoculated plants had bolted while the control (CP-MM), PVX/ FTΔCP sap and *in vitro* RNA inoculated plants remained in the vegetative phase at this stage.

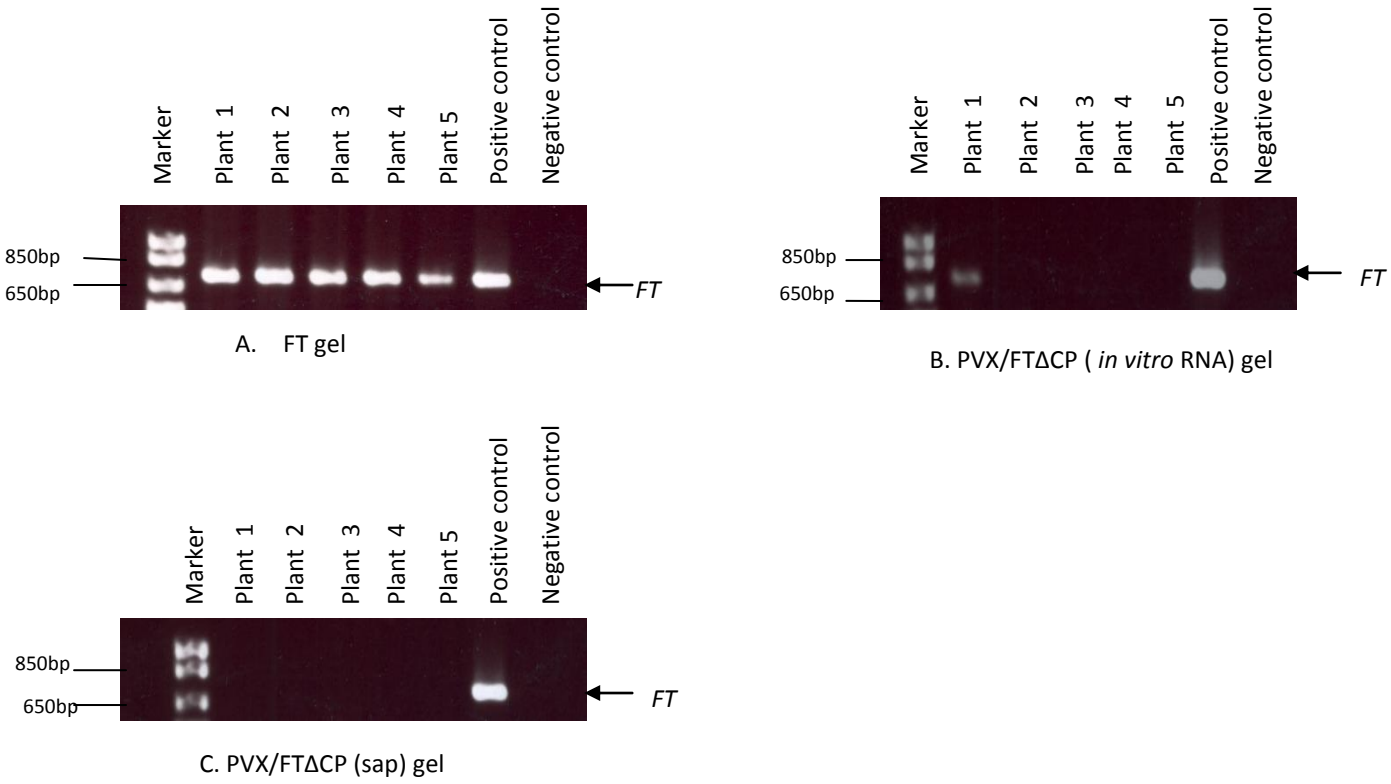


Figure 5.13 RT-PCR detection of virally expressed *Arabidopsis FT* RNA in systemic leaf tissue samples of inoculated CP-MM. tobacco plants. 5µl of 1kb plus ladder marker (invitrogen) was used. PVX specific forward primer PP82 (►) and gene specific reverse primer (PP356) was used.

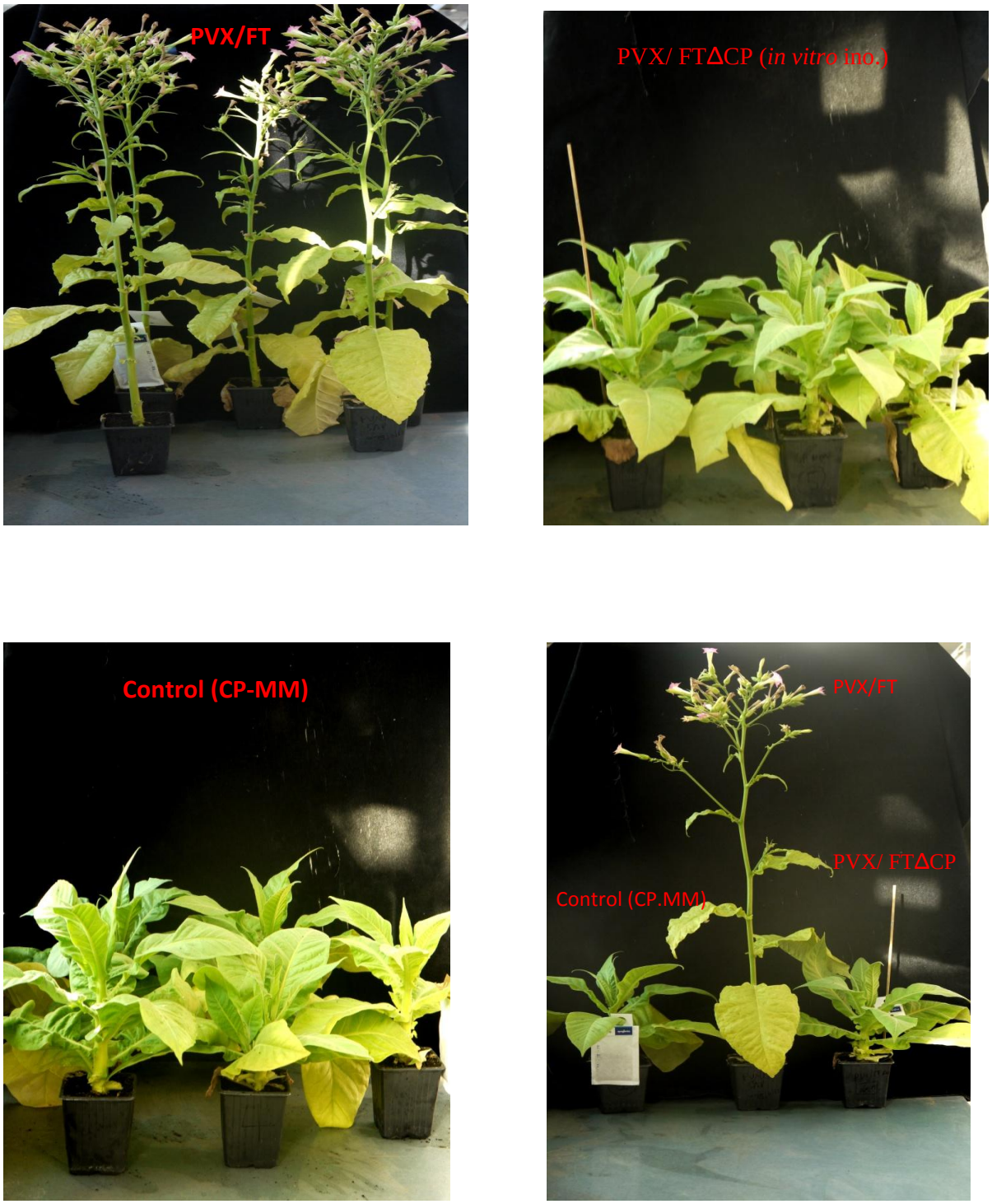


Figure 5.14. 40 days post inoculation of CP-MM plants. All PVX/FT inoculated plants flowered while the control (CP-MM), PVX/ FTΔCP sap and *in vitro* RNA inoculated plants remained in the vegetative phase.

Conclusion

The data presented in this chapter demonstrates that the virally expressed *Arabidopsis FT* and coat protein mRNA does not get transmitted into the germline/seeds of inoculated plants, therefore from a biocontainment perspective the viral expression system is a viable tool. RT-PCR test on systemic leaves harvested from CP-MM transgenic plants that was inoculated with PVX/FTΔCP *in vitro* RNA did confirm that the movement-deficient mutant PVX/FTΔCP transcripts were detected. This indicated that the movement- deficient PVX/FTΔCP RNA had undergone long distance movement from the initial site of inoculation to the vasculature which could only have occurred as a result of complementation of cell-cell competence in the movement- deficient PVX/FTΔCP by the intact CP transgene present in the tobacco plant. In relation to flowering time, the PVX/FTΔCP inoculated plants did not flower early compared to the PVX/FT plants. A plausible explanation for this occurrence could be due to low concentration of target RNA.

RT-PCR test from both preliminary and main experiments did confirm that the target RNA was expressed but there was a difference in band intensity between the PVX/FT and PVX/ FTΔCP *in vitro* RNA lanes as seen in Fig 5.9 and 5.13. The low levels of PVX/FTΔCP RNAs in systemic leaves suggested that there was a reduction in the efficiency of complementation by the viral CP expressed from the 35S promoter in the CP-MM transgenic plants which was most likely due to slower rate of *in vivo* encapsidation of the movement deficient PVX/ FTΔCP RNA in contrast to the PVX/FT inoculated plants which flowered early due to the presence of its fully functional CP that caused cell -to- cell movement, systemic spread and eventual movement of FT into the SAM where it triggers flowering. This explains why the PVX/ FTΔCP inoculated plants flowered late because a certain threshold level of RNA concentration is needed in order to induce any significant effect on flowering.

Chapter 6
General discussion

Chapter 6: General Discussion

6.1 General Discussion

6.1.1 *FT*, a mobile floral stimulus

Flowering in plants is essential for reproductive success and production of offspring. To maximize the chance of success and the survival of offspring the transition takes place at an appropriate time of the year. As described in chapter 1, there are seven main pathways which involve many interacting genes in the regulation of flowering. Molecular and genetic approaches have made it possible to understand the major role that *Arabidopsis FT* and its homologues play in the regulation of floral induction (Wigge *et al.*, 2011). Under inductive conditions, the transcription of *FT* is upregulated by CO in the leaf, the FT protein moves from the leaf to the shoot apical meristem (SAM) via the phloem and induces flowering (Corbesier *et al*., 2007). There is no doubt that FT protein is an essential component of florigen that triggers the induction of flowering. There are still many outstanding questions about the role of FT and the nature of florigen, for example how FT levels are controlled by many different endogenous and environmental signals. It would also be interesting to know if FT is transported alone or as part of a complex with other compound(s) from the leaves to the shoot apical meristem. Another point of interest would be to uncover the precise mechanism involved in controlling this movement. Understanding the mechanism could also shed more light on other possible signalling pathways.

6.1.2 *FT* mRNA acts as a long–distance mobile molecule

A few papers have reported the failure to detect systemic movement of *FT* mRNA. For example in grafting experiment, Lifschtz *et al.* (2006) reported that they could not detect the presence of *SFT* mRNA in the plant's shoots even though the transcripts were detected in the 35S: *SFT* donor scion which led to the conclusion that *SFT* mRNA was not mobile. They suggested that *SFT* protein and not *SFT* mRNA was acting as a mobile signal in the plant to induce flowering.

In addition, studies in *Brassica napus*, which is a close relative to *Arabidopsis thaliana*, revealed that FT protein was identified amongst other signalling proteins during the analysis

of soluble fractions of the sieve-tube exudates from *brassica* phloem sap although the possibility of a role of *FT* mRNA as part of the florigenic signal that moves from the leaf to the shoot apex to induce flowering was not ruled out. (Giavalisco *et al*., 2006).

A year later Corbesier *et al.* (2007) generated *Arabidopsis* and rice transgenic plants that expressed *FT*: *GFP* fusion gene controlled by the phloem specific promoter *SUC2*. It was reported that *FT:GFP* mRNA was detected in the phloem tissue but not at the SAM. In addition Lin *et al.* (2007) reported that grafting experiment in cucurbits showed the ability of FT protein to move in the phloem tissue through to the SAM where it triggers flowering. Tamaki *et al.* (2007) did report that both the FT protein as well as *FT* mRNA could be detected in the SAM of rice but at low levels. Data presented in this thesis using a non-translatable mutant version of *FT* (m*FT*) demonstrated that the *FT* mRNA is capable of long distance movement and is also able to move systemically. These results were confirmed in multiple repeat experiments in both tobacco and tomato plants. As both FT protein and *FT* mRNA have been confirmed to be involved in long distance movement, one cannot exclude the possibility of an interaction between FT protein, *FT* mRNA and possibly other host factors and compounds forming a mobile protein-RNA complex which ultimately triggers flowering.

6.1.3 *FT* plays different roles in plant growth and development

Remarkable progress has been achieved in understanding the role that *FT* plays in floral transition. Various reports have also shown that *FT* plays other roles particularly in plant growth and development for example apart from being involved in floral induction, the tomato *FT* homologue *SFT* plays a role in leaf architecture and stem growth. In *Arabidopsis* as well as triggering flowering, *FT* plays a role in meristem maintenance and also stomatal opening. In potato, the *FT* paralogues *StSP6A* and *StSP3D* have key roles in tuberisation and floral induction respectively. In this project, I have shown that tomato *FT* genes have different roles. In tomato, the overexpression of tomato *FT* (*SP2I, SP5G*) and *FT* caused increased seed production. Interestingly tomato *SP6A* did not have a significant effect on seed production but did have an effect in triggering early flowering in brassica (broccoli). In addition the overexpression of the tomato *FT* genes, *Arabidopsis FT* and tagged *FT* in tomato

caused increased branching. These findings were complemented by reports presented by other research groups e.g the rice *FT* (*Hd3a*) was shown to have a role in branching in potato (unpublished) while *FT* and *TSF* were shown to have roles in branching in *Arabidopsis* (Hiraoka *et al*., 2012). There is no doubt that *FT* plays multifunctional roles in different plant species as highlighted in this project.

6.2 Potential Commercial application of plant virus vector systems

In recent years the expression of heterologous genes in plants has played an important role in plant biotechnology. Plant virus vector systems are currently being utilised to a much greater extent due to the low cost, high level of heterologous protein production and speed of process compared to plant transgenic technology (Touriño *et al*., 2008). The PVX vector in particular is relatively stable and possesses mechanical transmissibility to a number of solanaceous plants as described in chapter 1. An added advantage of this virus is that the expressed viral mRNA does not get transmitted into the germline of inoculated plants thus making it a suitable tool for plant breeding.

Plant virus vector systems have also been used in the production of pharmaceutical proteins for example the human interferon αD gene was expressed in CaMV in brassica host plants. Large-scale production has however been yet to be exploited. Another example is the utilisation of the TMV vector in the production of biologically functional α-trichosanthin protein which is a potent inhibitor of HIV replication (Scholthof *et al*., 1996). CPMV has also been used for the production of antigenic peptide molecules against human rhinovirus14 and HIV (Lomonossoff *et al*., 2000). These peptide molecules are administered to animals such as rabbits, goat and mice to stimulate the production of specific antibodies which could be used as diagnostics tools in the detection of these diseases.

Plant virus vector systems are also useful tools in studying gene function. In my research project plant virus vectors were used to study long distance movement of *FT* mRNA in tobacco and tomato. It was also used to discover the functions of *FT* orthologues in different plant species. Experimental results from this project has demonstrated that with the utilisation of plant virus vector systems it is possible to significantly reduce breeding times by shortening the vegetative phase growth therefore it could be advantageous to plant breeders

because the time to breed finished varieties could be shortened significantly and also more seeds could be harvested for subsequent research or storage if needed.

6.3 Further work

FT plays a crucial role in floral induction. It integrates the inputs from a complex network of flowering signalling pathways. By utilising plant virus based RNA mobility assays I have shown that *FT* mRNA is able to move systemically and it is capable of long distance movement. I have also shown that the tomato *FT* (*SP6A*) is involved in inducing early flowering in *Brassica oleracea* (broccoli). In tomato, the overexpression of *FT* and *FT* orthologues caused increased seed production and lateral side shoot development. The exact mechanism of action of these genes in the control of seed production and lateral side shoot development is unclear.

Follow up experiments could be carried out in order to investigate the effects of *FT* expression on other plant species e.g. barley, onion, pepper using other plant virus vector systems. For example *FTC4* expression in tobacco caused an increased seed production and triggered early flowering but it did not have such a significant effect on flowering time and seed productivity in tomato therefore it would be interesting to investigate if that *FT* construct would have any effect on other plant species. In potato *StSP6A* and *StSP3D* have both been documented to have a role in tuberisation and flowering respectively. Other virus vector systems could be utilised to express for example the *StSP6A* in onions and test its effect in bulbing or *StSP3D'*s effect on flowering time in tomato.

In relation to lateral side shoot development (branching), an enzyme assay could be carried out to investigate the endogenous levels of the plant's hormones throughout the plant's developmental stages during an experiment in order to test if there is any correlation with the virally expressed target gene and hormonal levels. Tagged FT constructs were used in order to study the floral induction functionality and *in vivo* distribution of the protein within the plant. Protein-protein interaction/binding could be studied by carrying out a pull down assay to identify other component of the florigen complex. X-ray crystallography could also be carried out on the wild-type FT, mutant FT proteins and FT orthologues in order to study the structural framework of the proteins.

In the investigation to generate a potential environmental biocontainment technology, the CP transgenic experiment investigated whether the CP-MM tobacco plants could complement the CP mutated movement deficient construct; PVX/ FTΔCP. Further work could be implemented by carrying out repeated infection on host plant. Infection with two independent viruses would produce a synergistic effect and several-fold increase of the viral RNA. Another possibility could be increasing the concentration of *in vitro* transcribed *PVX/ FTΔCP* RNA used for inoculation of test plants.

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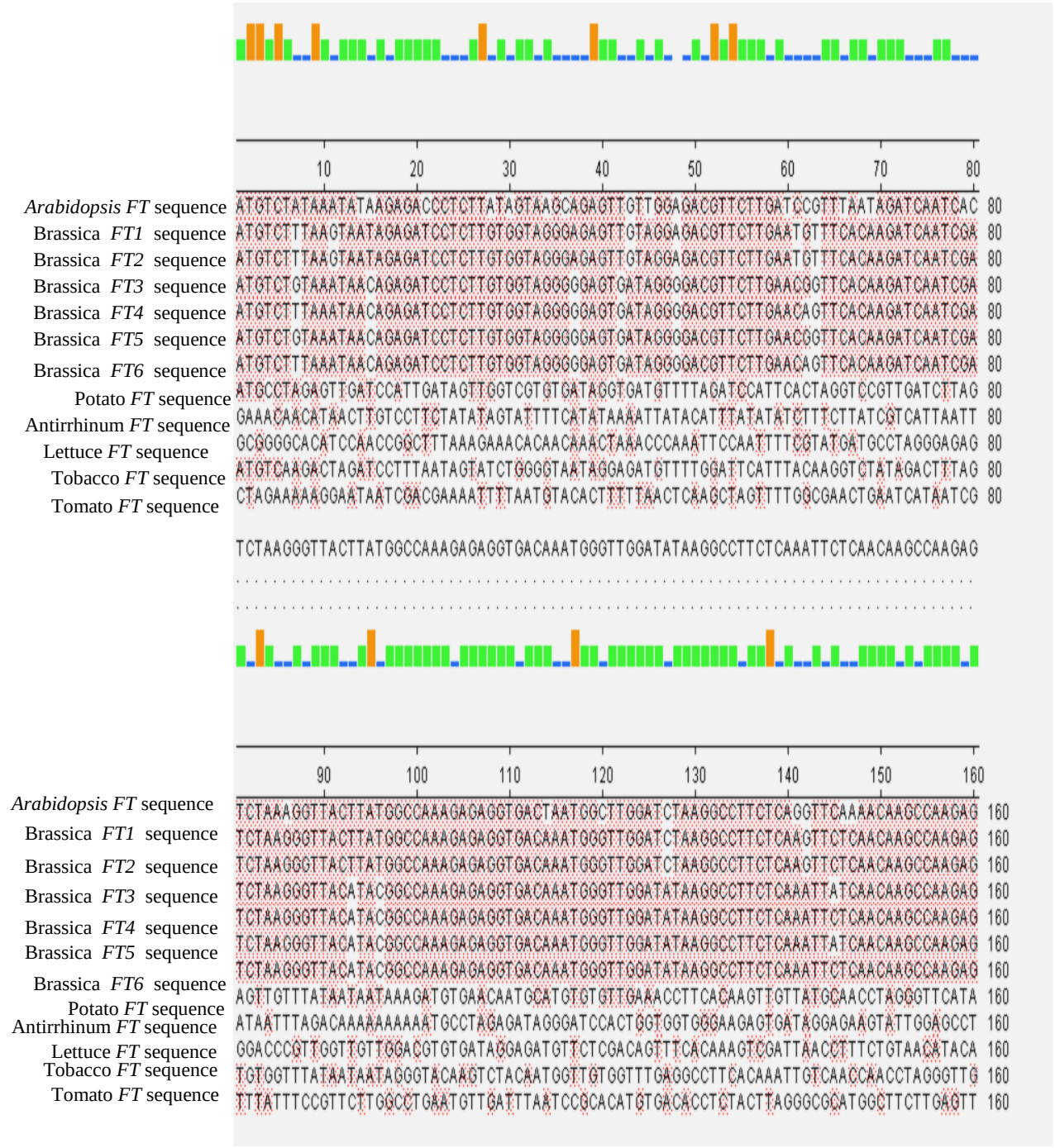
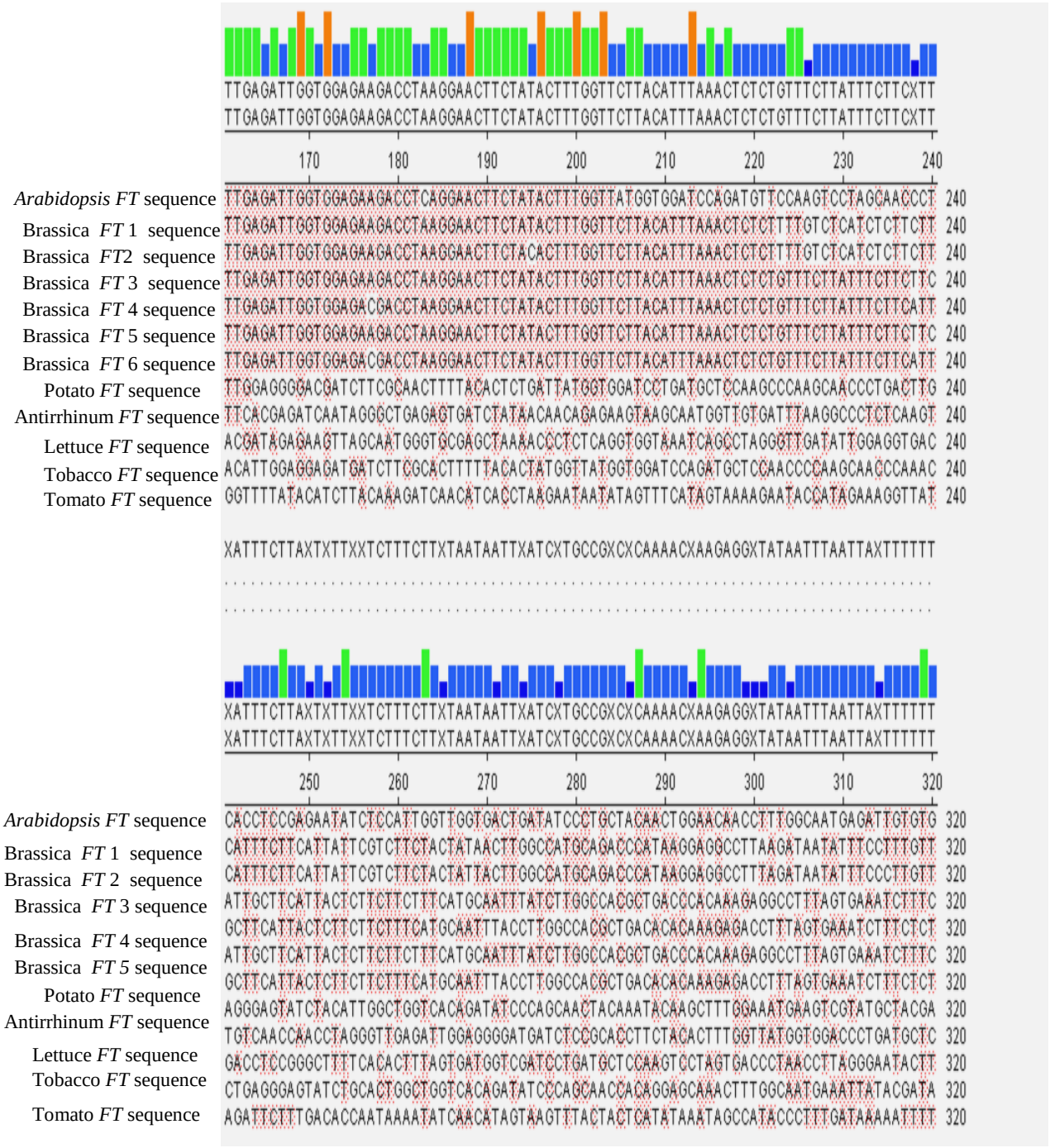
Appendix I Primers list

Primers	Sequence (5'-3')	Origin
PP82	CAGTGTTGGCTTGCAAACTAG	PVX
PP356	AGGAAGAAGTCGACTAAAGTCTTCTTCCTCCGCAG	*FT*
TCPF	GCCGGTACCATGTCAGCACCAGCTAGCA	*CP*
TCPR	GGT GTCGACTTATGGTGGTGGTAGAGTGACAA	*CP*
AttB1CPF	GGGGACAAGTTGTACAAAAAAGCAGGCTTTATGTCAGCAC CA CTAGAC	*CP*
AttB2CPR	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATGGTGGT GGTAGAGTGACAAC	*CP*
His-FTF	GGC CGGCCG ATGCAT CAT CAC CAT CACCACTCTAT AAATATAAGA	*FT*
His-FTR	GGCGTCGACCTAAAGTCTTCTTCCTCCGCAG	*FT*
FT-HisR	GCG GTCGACCTA GTG GTGATG GTG ATGATG AAGTCTTCTTCCTCCGC	*FT*
FT-HisF	GGCCGGCCG ATGTCTAT AAATATAAGA	*FT*
FT-FLAGR	GCG GTCGACCTACTT GTCATCGTCATCCTTGTAGTC AAGTCTTCTTCCTCCGCAG	*FT*
CPsF	TTGGGACTTAGT	*CP*
CPsR	ATTGCTGCTGC	*CP*
EFNBF	CTCCAAGGCTAGGTATGATG	*EF-1* α
EFNBR	CTTCGTGGTTGCATCTCAAC	*EF-1* α
SP2 F	GGC C G G C C G ATGGAAACTT CGGCGAGG	*SP2I*
SP2R	GCG G T C G A C CTAGTGATGAGCAGCAT	*SP2I*
SP5F	GGCCGGCC G ATGCCTAGAGATCCTTTAATAGTTTCT	*SP5G*

SP5R	GCG G T C G A C TTATAGGCGACGACCACC	*SP5G*
SP6F	C G G C C G ATGCCTAGAGTTGATCCATTGAT	*SP6A*
SP6R	GCGGTCGACTTAGAAATTTTG	*SP6A*

Appendix II ***FT*** **orthologues sequence alignment**




Arabidopsis FT sequence
Brassica FT 1 sequence
Brassica FT2 sequence
Brassica FT 3 sequence
Brassica FT 4 sequence
Brassica FT 5 sequence
Brassica FT 6 sequence
Potato FT sequence
Antirrhinum FT sequence
Lettuce FT sequence
Tobacco FT sequence
Tomato FT sequence
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190
200
210
220
230
240
250
260
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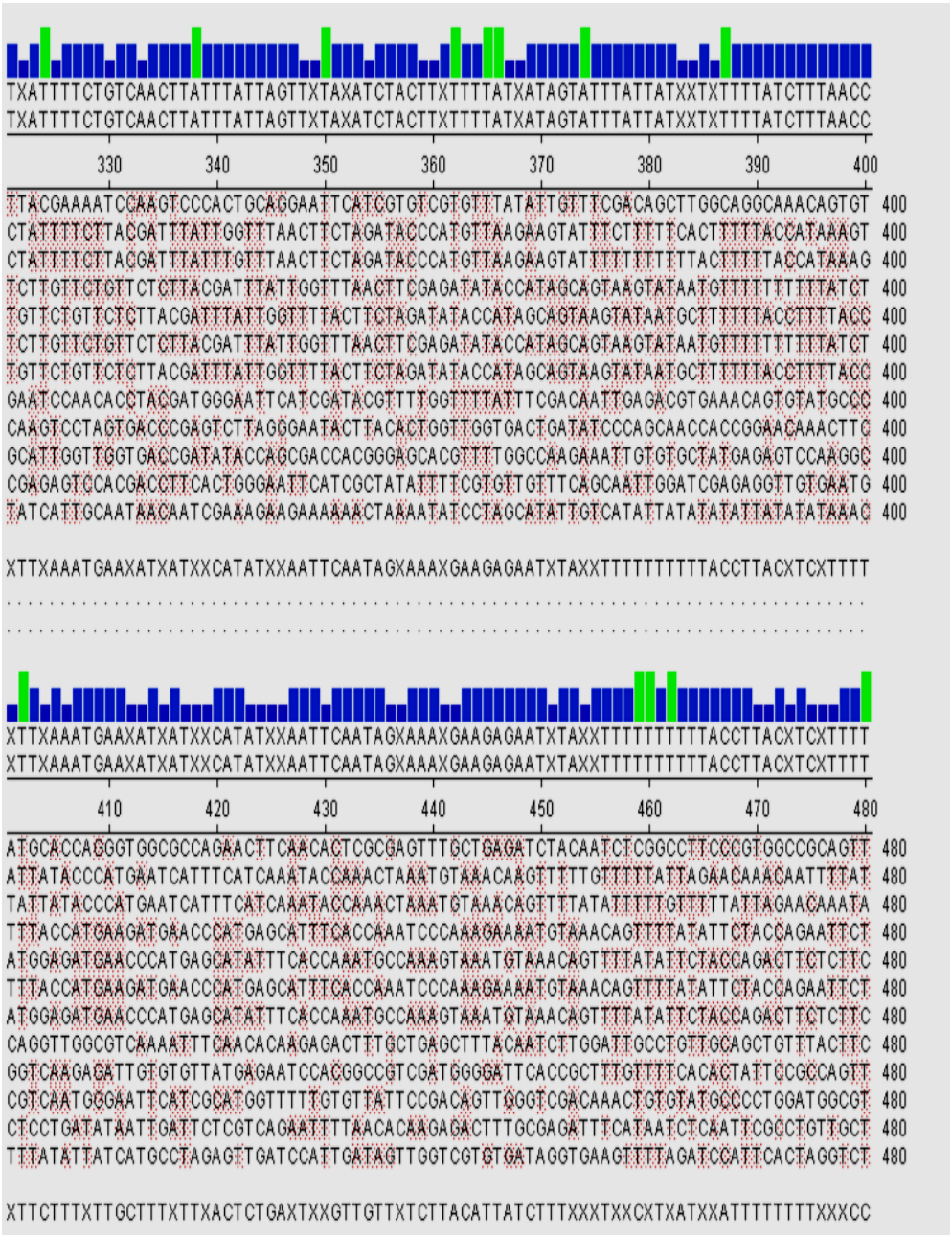
290
300
310
320


Arabidopsis FT sequence
Brassica FT 1 sequence
Brassica FT 2 sequence
Brassica FT 3 sequence
Brassica FT 4 sequence
Brassica FT 5 sequence
Potato FT sequence
Antirrhinum FT sequence
Tobacco FT sequence
Lettuce FT sequence
Tobacco FT sequence
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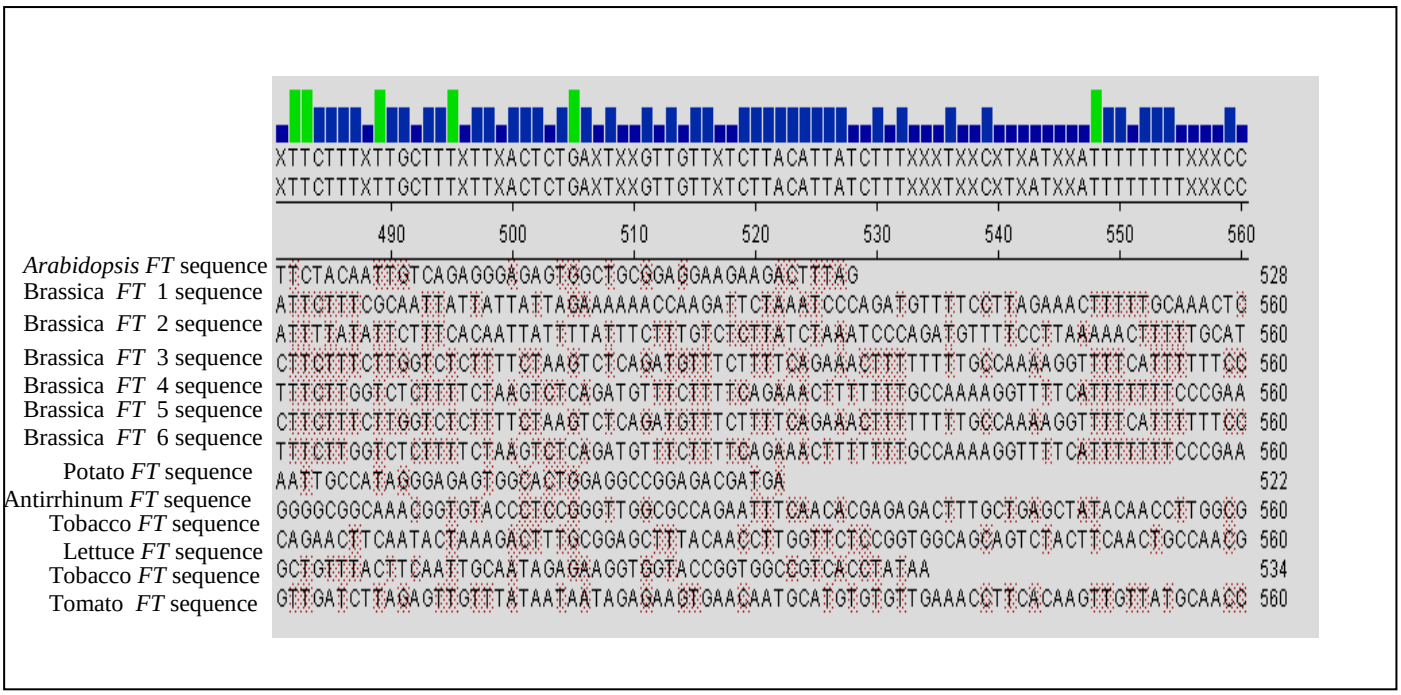
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XTTCTTTXTTGCTTTXTTXACTCTGAXTXXGTTGTTXTCTTACATTATCTTTXXXTXXCXTXATXXATTTTTTTTXXXCC
490
500
510
520
530
540
550
560
Arabidopsis FT sequence TTCTACAATTGTCAGAGGGAGAGTGGCTGCGGAGGAAGAAGACTTTAG 528
Brassica FT 1 sequence ATTCTTTCGCAATTATTATTATTAGAAAAAACCAAGATTCTAAATCCCAGATGTTTTCCTTAGAAACTTTTTGCAAACTG 560
Brassica FT 2 sequence ATTTTATATTCTTTCACAATTATTTTATTTCTTTGTCTCTTATCTAAATCCCAGATGTTTTCCTTAAAAACTTTTTGCAT 560
Brassica FT 3 sequence CTTCTTTCTTGGTCTCTTTTCTAAGTCTCAGATGTTTCTTTTCAGAAACTTTTTTTTGCCAAAAGGTTTTCATTTTTTCC 560
Brassica FT 4 sequence TTTCTTGGTCTCTTTTCTAAGTCTCAGATGTTTCTTTTCAGAAACTTTTTTTTGCCAAAAGGTTTTCATTTTTTTCCCGAA 560
Brassica FT 5 sequence CTTCTTTCTTGGTCTCTTTTCTAAGTCTCAGATGTTTCTTTTCAGAAACTTTTTTTTGCCAAAAGGTTTTCATTTTTTCC 560
Brassica FT 6 sequence TTTCTTGGTCTCTTTTCTAAGTCTCAGATGTTTCTTTTCAGAAACTTTTTTTTGCCAAAAGGTTTTCATTTTTTTCCCGAA 560
Potato FT sequence AATTGCCATAGGGAGAGTGGCACTGGAGGCCGGAGACGATGA 522
Antirrhinum FT sequence GGGGCGGCAAACGGTGTACCCTCCGGGTTGGCGCCAGAATTTCAACACGAGAGACTTTGCTGAGCTATACAACCTTGGCG 560
Tobacco FT sequence CAGAACTTCAATACTAAAGACTTTGCGGAGCTTTACAACCTTGGTTCTCCGGTGGCAGCAGTCTACTTCAACTGCCAACG 560
Lettuce FT sequence GCTGTTTACTTCAATTGCAATAGAGAAGGTGGTACCGGTGGCCGTCACCTATAA 534
Tobacco FT sequence GTTGATCTTAGAGTTGTTTATAATAATAGAGAAGTGAACAATGCATGTGTGTTGAAACCTTCACAAGTTGTTATGCAACC 560
Tomato FT sequence